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TITLE: Compositions and methods for reducing radiation and drug resistance in cells

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INVENTOR-INFORMATION:

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This application is related to provisional application Ser. No. 60/034,160, filed Dec. 30, 1996, to which priority is claimed.

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PRIOR-ART-DISCLOSED:

U.S. PATENT DOCUMENTS

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	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	5576208	November 1996	Monia et al.	435/240.2
☐	5599704	February 1997	Thompson et al.	435/325
☐	5734039	March 1998	Calabretta et al.	536/24.5

FOREIGN PATENT DOCUMENTS

FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
WO 95/32987	December 1995	WO	

OTHER PUBLICATIONS

Denner, Ia et al. (Jul. 21, 1994) WO9415645-A. Database: Geneseq, Q67036 (Accessed Jul. 24, 1998).
Kasid et al. Science, Mar. 10, 1989. vol. 243, pp. 1354-1356.
Daum et al. TIBS 19, 474-480 (Nov. 1994).
Tseng et al. Cancer Gene Therapy. vol 1 (1), pp. 65-71 (Mar. 1994).
Branch, A.D. TIBS 23, 45-50 (Feb. 1998).
Gewirtz et al. PNAS. 93, 3161-3163 (Apr. 1996).
Rojanasakul. Advanced Drug Delivery Review. 18, 115-131 (Jan, 30, 1996).
Maher et al. Archives of Biochemistry and Biophysics. Vol. 253, pp. 214-220 (Feb. 15, 1987).
Gura, T. Science, vol. 278, pp. 1041-1042 (Nov. 1997).
Crooke, S.T. Chapter 1, Basic Principles of Antisense Therapeutics on "Antisense Research And Application" (ed. Stanley T. Crooke), Springer-Verlag, New York, (Jul. 1998) pp. 1-50.
Betram, J., et al., "Reduction of erbB2 gene product in mamma carcinoma cell lines by erbB2 mRNA-specific and tyrosine kinase consensus phosphorothioate antisense oligonucleotides", (1994) Biochem. Biophys. Res. Commun. 200:661-667.
Bradley, Matthews O., et al., "Reversal of Transformed Phenotypes by Antisense fos", (1992) Annals New York Academy of Sciences, pp. 124-135.
Daum, Gunter, et al., "The ins and outs of Raf kinases", (1994) Trends Biochem. Sci. 19, 474-480.
Dean, N.M., et al., "Antisense oligonucleotides as inhibitors of signal transduction: development from research tools to therapeutic agents", (1996) Biochem. Soc. Trans. 24, 623-629.
Kasid, U., et al., "Effect of Antisense c-raf-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma", (1989) Science 243, 1354-1356.
Kasid, U., et al., "The raf Oncogene is Associated with a Radiation-Resistant Human Laryngeal Cancer", (1987) Science 237, 1039-1041.
Kizaka-Kondoh, Shinae, et al., "Raf-1 Protein Kinase Is an Integral Component of the Oncogenic Signal Cascade Shared by Epidermal Growth Factor and Platelet-Derived Growth Factor", (1992) Mol. Cell. Biol. 12:5078-5086.
Ledwith, Brian J., et al., "Antisense-fos RNA Causes Partial Reversion of the Transformed Phenotypes Induced by the c-Ha-ras Oncogene", (1990) Mol. Cell. Biol. 10:1545-1555.
Sepp-Lorenzino, Laura, et al., "Signal transduction pathways induced by heregulin in MDA-MB-453 breast cancer cells", (1996) Oncogene, 12, 1679-1687.
Soldatenkov, MD, V.A., et al., "Inhibition of Raf-1 Protein Kinase by Antisense Phosphorothioate Oligodeoxyribonucleotide Is Associated with Sensitization of Human Laryngeal Squamous Carcinoma Cells to Gamma Radiation", (1997) The Cancer Journal from Scientific American 3, 13-20.
Suy, Simeng, et al., "Association of Grb2 with Sos and Ras with Raf-1 upon gamma irradiation of breast cancer cells", (1997) Oncogene, 15, 53-61.
Vaughn, James P., et al., "Inhibition of the erbB-2 tyrosine kinase receptor in breast cancer cells by phosphoromonothioate and phosphorodithioate antisense oligonucleotides", (1996) Nucleic Acids Res., 24, 4558-4564.

ART-UNIT: 165

PRIMARY-EXAMINER: Brusca; John S.

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ABSTRACT:

Provided are antisense oligonucleotides directed against the raf-1 gene, Ha-ras gene and HER-2 gene, components of a signal transduction pathway involving oncogenes and their normal counterparts and leading to the phenotype of cellular radioresistance. Administration of these antisense oligonucleotides is shown to reverse the radioresistance phenotype in cells overexpressing HER-2 or a mutant form of Ha-ras. Methods and compositions for reversing radiation resistance among other conditions involving these genes are disclosed.

12 Claims, 6 Drawing figures

Exemplary Claim Number: 1
Number of Drawing Sheets: 7

BRIEF SUMMARY:

1 The failure of a significant number of tumors to respond to drug and/or radiation therapy is a serious problem in the treatment of cancer. While the genetic basis of this resistance in mammalian cells is still poorly understood, evidence has been obtained in recent years linking proto-oncogenes and oncogenes to the phenomenon of cellular radiation resistance.

2 The earliest report of such a possible link was that of FitzGerald et al. in 1985 [FitzGerald, T. J. et al. (1985) Am. J. Clin. Oncol. 8: 517-522], who found that transfection of NIH 3T3 cells with a human N-ras oncogene was able to increase the radiation resistance level of the recipient cell line. Expanding upon this was the report by Sklar [Sklar, M. D. (1988) Science 239: 645-647] that NIH 3T3 cells transformed not only by N-ras but also by mutated Ha- and Ki-ras were more radiation resistant than the parent cell line. Additionally, we demonstrated a similar effect on the radiation resistance level of NIH 3T3 cells by both the mutated form of Ha-ras and the overexpression of the Ha-ras proto-oncogene [Pirollo, K. F. et al. (1989) Int. J. Radiat. Biol. 55: 783-796]. A synergistic increase in the radiation resistance level of primary rat embryo cells was also seen after cotransfection of ras and myc oncogenes [Ling, C. C. and B. Endlich (1989) Radiat. Res. 120: 267-279; McKenna, W. G. et al. (1990) Cancer Res. 20: 97-102]. All documents cited herein supra and infra are hereby incorporated in their entirety by reference thereto.

3 The association was extended to include other oncogenes when transfection of NIH 3T3 cells by high molecular weight DNA from both radiation-resistant cells derived from a human laryngeal squamous carcinoma [Kasid, U. et al. (1987) Science 237: 1039-1041] and radiation-resistant noncancerous skin fibroblast (NSF) cell lines from members of a family with Li-Fraumeni syndrome [Pirollo et al., 1989, supra; Chang, E H. et al. (1987) Science 237: 1036-1039] led to the identification of an activated human raf-1 oncogene in the resulting radiation-resistant transformants. Transfections not only of the raf-1 oncogene but also of other protein-serine kinase encoding oncogenes, mos and cot, have been shown to confer the radiation-resistant phenotype on the recipient human Beas-2B [Kasid, U. et al. (1989) Abstract, 5th Annual Meeting on Oncogenes] mouse NIH 3T3 (Pirollo et al.,1985, supra) and hamster SHOK cells [Suzuki, K. et al. (1992) Radiat. Res. 129: 157-162], respectively. The effect of activated oncogenes on the radiation resistance level of NIH 3T3 cells is not a generalized phenomenon but is particular to specific oncogenes, as was clearly shown by Sklar [Sklar, 1988, supra; Sklar, M. D. et al. (1986) Int. J. Radiat. Oncol. Biol. Phys. 12: 190-191, Abstract] for abl and fms and by our laboratory [Pirollo et al., 1985, supra] for myc, fes, and abl.

4 Evidence continues to accumulate which indicates that the normal counterpart of many of the known oncogenes (proto-oncogenes) are involved in vital, normal cellular functions [Bishop, J. M. (1991) Cell 64: 235-248; Cantley, L. C. et al. (1991) Cell 64: 281-302; Hunter, T. (1991) Cell 64: 249-270]. They have also been shown to interact with one another as components of a proposed signal transduction pathway which involves transmission of messages from the membrane to the nucleus directing the cells to divide or to differentiate. On the basis of antibody-blocking experiments, it has been proposed that raf-1 is downstream of ras in this pathway [Morrison, D. K. (1990) Cancer Cells 2: 377-380; Rapp, U. R. et al. (1988) In The Oncogene Handbook, T. Curran, J. E. P. Reddy and A Skala, Eds, pp. 213-252. Elsevier, Amsterdam; Smith, M. R. et al. (1986) Nature 320: 540-543; Weinstein, I. B. (1988) Mutat. Res. 202: 413-420].

5 Part of the signal transduction pathway leading to raf-1 expression is HER-2 (c-erb B-2/neu) which encodes a transmembrane protein tyrosine kinase with extensive homology to the epidermal growth factor receptor (EGF-R). Elevation of HER-2 in cancer cells has been shown to correlate with failure to respond to

radiation therapy and there is significant evidence that expression of HER-2 affects the response of breast cancer tumors to endocrine therapy with Tamoxifen, and chemotherapy using drugs such as cisplatin, carboplatin, 5-fluorouracil, mitoxantrone, cyclophosphamide, methotrexate, doxorubicin, carmustine, melphalan, mitomycin, etoposide and combinations of these drugs [Pegram, M. D. et al. (1993) Proc. 84th Ann. Mtg. of AACR, Orlando, 19-22 May 1993 34, p26 (Abstract); Wright, C. et al. (1992b) Br. J. of Can. 65: 271-274; Allred, D. C. et al. (1992) J. Clin. Onc. 10: 599-605; Gusterson, B. A. et al. (1992) J. Clin. Onc. 10: 1049-1056; Van Diest, P. J. et al. (1988) Path. Res. Pract. 188: 344-349; Muss, H. B. et al (1994) NEJM 330: 1260-1266; Tsai, C.-M. et al. (1993) J. Natl. Can Inst. 85: 897-901].

6 Recent studies indicate that the radiation resistant (RR) phenotype appears to be linked to the activation of specific protooncogenes in a signal transduction pathway involving HER-2 as an upstream member of the pathway and Ha-ras and raf-1 downstream of HER-2, analogous to that described for cell growth and differentiation [Pirollo, K. F. et al. (1993) Rad. Res. 135:234-243]. We hypothesized that disruption of the pathway therefore should lead to reversal of this phenotype and increased sensitivity of resistant cell to drug/radiation therapy which would have far reaching clinical implications in the treatment of drug and radiation resistant tumors.

7 SUMMARY OF THE INVENTION

8 A specific strategy to interfere with the signaling is to modulate the expression of specific genes in the pathway at the RNA level using antisense oligonucleotides (ASO). Short antisense DNA oligonucleotides selectively bind to cellular mRNA targets through complementary sequence-specific Watson-Crick base pairing. The hydrogen-bonded antisense molecule can modulate the expression of the targeted gene product [Uhlmann, E. and Pyman, A. (1990) Chem. Rev. 90: 544-584]. We and others have demonstrated the ability of antisense oligonucleotides and their modified analogues to specifically inhibit ras p21 protein synthesis in in vitro translation, in cell culture, and in tumorigenesis in nude mice [Yu, Z. et al. (1989) J. Experim. Path. 4: 97-108; Brown, D. et al. (1989) Oncogene Res. 4: 243-252; Chang, E. H. et al. (1991) Biochemistry 30: 8283-8286; Ts'o, P. O. P. et al. (1992) Annals. N. Y. Aca. Sci. 660:150-177; Plenat, F. (1997) Mol. Med. Today 6: 225-267]. Additionally, ASO against genes such as c-myb, c-myc, c-fos, BCR-ABL and the IGF receptor, have also been shown to suppress human tumor cell growth in vitro and in some cases are currently in clinical trials as anti-cancer therapeutics [Stein, C.A. et al. (1988) Nucl. Acids. Res. 16: 3209-3221; Mercola, D. and Cohen, J. S. (1995) Cancer Gene Ther.2:47-59; Scanlon, K. J. et al. (1995) FASEB J. 9: 1288-1296].

9 The serine/threonine kinase Raf-1 protein appears to be a central component of multiple signal transduction pathways in the cell [Reviewed in: Campbell, J. S. et al. (1995) Rec. Prog. Hormone. Res. 50: 131-159; Daum, G. et al. (1994) TIBS 19: 474-480] including that for radiation resistance. Consequently, the use of ASO against raf-1 itself, or against upstream effectors of raf-1 such as Ha-ras and HER-2, to impede signaling through this gene should result in increased drug and radiation sensitivity, which would have far reaching clinical implications in the treatment of radioresistant tumors.

10 Therefore, the present invention relates to a method for reversing the drug and radiation resistance phenotype of cells, more specifically tumor cells which have acquired drug and/or radiation resistance. The method of the present invention employs antisense oligonucleotides targeted against specific proto-oncogenes in the signal transduction pathway leading to the radiation resistant phenotype. More specifically, the method employs the administration of antisense oligonucleotides complementary to unique sequences of at least one of raf-1, Ha-ras, and HER-2 genes such that the expression of these factors is reduced, and the cells are radiosensitized.

11 Therefore, it is an object of the present invention to provide antisense oligonucleotides for reverting radiation and drug resistant cells in vitro and

in vivo, for use in diagnostic assays for detecting expression of genes in the signal transduction pathway leading to radiation and/or drug resistance, and for use as therapeutic agents for inhibiting tumor cell growth to improve response to conventional therapeutics and therefore improve survival.

12 It is another object of the present invention to provide a method for decreasing raf-1, Ha-ras, or HER-2 expression important in reverting radiation resistant cells to radiation sensitive cells or to reduce symptoms of diseases resulting from the overexpression of these genes. For example, it is possible to inhibit restenosis, abnormal wound repair, or any biological activity which is produced by signaling through these pathways. These genes are involved in multiple signal transduction pathways, one of which, the MAPK pathway, is considered antagonistic to apoptosis. Perturbation of the signal transduction pathways by these antisense oligonucleotides may lead to or potentiate apoptosis. Ras signaling through the Ras/MAPK pathway may also play a role in formation of long-term memory and abnormal expression may therefore impact on disease states such as senility and Alzheimer's. Mutated Ras acting through a different signaling pathway, inhibits skeletal muscle differentiation. A mutation in a member of the raf family, A-raf, was found in mice to lead to neurological and gastrointestinal abnormalities and death in mice. Elevated levels of a fragment of HER-2 protein found in sera was considered a possible cause of pre-eclampsia or HELLP syndrome in pregnant women. Therefore, decreasing expression of these genes may be important in reversing or reducing these conditions and diseases.

13 It is a further object of the present invention to provide a method to resensitize radiation- and drug-resistant cells, the method comprising administering to the cells antisense oligonucleotides of genes identified in the signal transduction pathway leading to resistance such as oligonucleotides of the present invention. Other genes involved in the MAPK signal transduction pathway are defined in FIG. 6. Applicants have found that growth factor sis (PDGF-.beta.), receptor tyrosine kinases trk (nerve growth factor), met (hepatocyte growth factor), tyrosine kinase src, serine/threonine kinase mos, protein kinase C .beta.-1, nuclear oncogene ets-1, as well as some other components of the MAPK pathway, are involved in the radiation resistant phenotype, and administration of oligonucleotides which block the transduction pathway through these and other genes in this pathway may reduce the radiation resistance phenotype [Pirollo, K. F. et al. (1993) Rad. Res. 135:234-243]. This method is important in the treatment of tumors, especially tumors which have acquired resistance to radiation and drugs, both endocrine and chemical.

14 It is yet another object of the present invention to provide a method for inhibiting tumor growth by reducing levels of raf-1, Ha ras or HER-2 in said tumor, thereby resensitizing radiation- and drug-resistant cells in the tumour to radiation and drugs such that these cells can be treated again with either radiation or drugs.

15 It is still another object of the present invention to provide a method for detecting the level of raf-1, Ha-ras or HER-2 RNA in a cell comprising labeling the antisense oligonucleotides of the present invention and using the labeled oligonucleotides in a hybridization assay or a polymerase chain reaction (PCR) assay to detect the presence of the gene or amount of raf-1, Ha-ras and HER-2 RNA in a cell.

16 It is yet another object of the present invention to provide a therapeutic agent for treating diseases associated with an increase in raf-1, Ha-ras and HER-2, such as cancer, for example, the agent comprising the antisense oligonucleotides of the present invention in a pharmaceutically acceptable amount, in a pharmaceutically acceptable excipient.

17 It is further another object of the present invention to provide a method for visualizing raf-1, Ha-ras and HER-2 RNA in an organism, said method comprising labeling the antisense oligonucleotides of the present invention with a detectable label useful for imaging, and administering the labeled oligonucleotides at the site where imaging is desired, and detecting the label.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

FIG. 1. The effect of anti-raf-1 oligonucleotides on Raf-1 p 72 protein synthesis and the radiation resistance levels of SCCHN cell lines JSQ-3 and SQ-20B. A--Western blot analysis of Raf-1 protein synthesis inhibition by increasing concentrations of raf-1 oligonucleotides. C=untreated cells; O=cells treated with liposomes but no oligonucleotides; AS=antisense; S=sense. B--Histogram demonstrating radiosensitization with increasing concentrations (0.1, 0.3, 1 .mu.M) of anti-raf-1 ASO. As controls, the cells were treated with 1 .mu.M of either a sense or a scrambled raf-1 oligonucleotide. Radioresistance levels are given as D.sub.10 values. Error bars represent the standard error of the mean (S.E.M.) of 2 to 13 values.

FIG. 2. Histogram demonstrating the effect of 1 .mu.M anti-raf-1 ASO on SK-OV-3, T24 and MCF10A cells. As controls, the cells were treated with 1 .mu.M of either a sense or a scrambled raf-1 oligonucleotide. Radioresistance levels are given as D.sub.10 values. Error bars represent the S.E.M. of 2-6 values.

FIG. 3. Survival curves, after graded doses of .gamma.-radiation, for A--JSQ-3 and B--SK-OV-3 cells untreated or treated with either 1 .mu.M raf-1 antisense or sense oligonucleotides. Curves are plotted as the log of the surviving fraction vs. radiation dose in Gy. Points are plotted as the S.E.M. of 2-13 values.

FIG. 4. Histogram demonstrating radiosensitization by Ha-ras ASO. The concentration of antisense ras oligonucleotide used to treat SK-OV-3 and MCF10A was 3 .mu.M. As controls, the cells were treated with 3 .mu.M of two different scrambled Ha-ras oligonucleotides. Radioresistance levels are given as D.sub.10 values. Error bars represent the S.E.M. of 2-7 values.

FIG. 5. The effect of anti-HER-2 oligonucleotides on p185 HER-2 protein synthesis and the radiation resistance levels of SK-OV-3, T24 and MCF10A cells. A--Western blot analysis of HER-2 protein synthesis inhibition by increasing concentrations (0.3, 1.0 & 3.0 .mu.M) of HER-2 oligonucleotides. C=untreated cells; O=cells treated with liposomes but no oligonucleotides; AS=antisense; S=scrambled. B--Histogram demonstrating radiosensitization by HER-2 ASO. The concentration of antisense HER-2 oligonucleotide used to treat T24 and MCF10A was 1 .mu.M. As controls, the cells were treated with 1 .mu.M of two different scrambled HER-2 oligonucleotides. Radioresistance levels are given as D.sub.10 values. Error bars represent the S.E.M. of 2-7 values.

FIG. 6. Raf-dependent signal transduction. For clarity, additional pathways, such as JAK/STAT-mediated signalling, are omitted, as are feedback phosphorylation reactions. Raf is activated upon stimulation of a variety of receptors. Together with MAP/ERK kinase (MEK) and mitogen-activated protein kinase (MAPK; also known as extracellular-receptor-activated protein kinase, ERK) it forms the highly conserved cytoplasmic kinase cascade. MAPK acts on numerous effector molecules, such as other serine/threonine kinases or transcription factors, which finally determine the cellular response. Taken from Daum, G. et al. (1994) Trends in Biochemical Sciences 19:474-480.

DETAILED DESCRIPTION:

1 DETAILED DESCRIPTION

2 As was discussed above, previous studies have indicated the presence of a

signal transduction pathway leading to cellular radiation resistance. We hypothesized that inhibiting or reducing the expression of members of this pathway in cells should block signaling leading to decreased radioresistance. Members of this pathway include, but are not limited to, raf-1, Ha-ras and HER-2. One method for reducing the expression of these genes is through antisense oligonucleotides or ribozymes.

3 Therefore, in one embodiment, the present invention relates to a composition of matter consisting essentially of at least one antisense oligonucleotide substantially complementary to an RNA sequence (mRNA or pregenomic RNA) encoded by the raf-1 gene, preferably at or near the initiation codon of raf1, at about nucleotides 130 to 147, or around the promoter sequence or at single stranded loops based on secondary structure. The oligonucleotide is preferably comprising a sequence of at least about 8 nucleotides, is preferably not more than about 40 nucleotides, more preferably about 15-20 nucleotides, and optimally about 18 nucleotides.

4 The present invention also relates to antisense oligonucleotides substantially complementary to an RNA sequence (mRNA or pregenomic RNA) encoded by the Ha-ras gene, preferably at or near the initiation codon of Ha-ras, at about nucleotides 1670 to 1680, or alternatively, near a region of the gene where mutations causing activation are most prevalent, for example at codons 12 and 61. The oligonucleotide is preferably comprising a sequence of at least about 8 nucleotides, is preferably not more than about 40 nucleotides, more preferably about 15-20 nucleotides, and optimally about 11 nucleotides.

5 The present invention also relates to antisense oligonucleotides substantially complementary to an RNA sequence (mRNA or pregenomic RNA) encoded by the HER-2 gene, preferably at or near the initiation codon of HER-2, at about nucleotides 708 to 718, or around the promoter sequence or at single stranded loops based on secondary structure. The oligonucleotide is preferably comprising a sequence of at least about 8 nucleotides, is preferably not more than about 40 nucleotides, more preferably about 15-20 nucleotides, and optimally about 11 nucleotides

6 As used herein, "substantially complementary" means that an antisense oligonucleotide of the invention is capable of hybridizing with its RNA target under physiological conditions, e.g., as pertains inside a cell expressing raf-1. Since there is substantial homology between the raf-1 family members, it is possible to use sequences from other family members as antisense nucleotides or to design other antisense sequences in order to inhibit their gene expression. Whether or not a sequence is substantially complementary can be determined by techniques known to those with ordinary skill in the art. For example, the sequence of the antisense oligonucleotide can be compared to the raf-1 sequence in EMBL databank, accession no. X03484, [Bonner, T. J. (1986) Nucl. Acids Res. 14:1009-1015], to the Ras sequence VOO574, J00276, J00277, K00954 [Capon, D. J. (1983) Nature 302: 33-37] and to the HER-2 sequence [Coussens, L. (1985) Science 230: 1132-1138] and its ability to hybridize to RNA under appropriate stringency conditions can be determined. Hybridization techniques are known in the art. See for example, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y.

7 As used herein, "consisting essentially of" has its usual meaning, i.e., that one or more compositions of matter of the invention may be used together, either in admixture or combined in a single molecule, with other materials that do not alter the essential nature of the invention. For example, while the antisense oligonucleotide sequences of the invention are essential to the invention, it is contemplated that they may be used in admixture or in chemical combination with one or more other materials, including other oligonucleotides, materials that increase the biological stability of the oligonucleotides, or materials that increase their ability to selectively penetrate their target cells and reach and hybridize to their target RNA. Furthermore, the term "oligonucleotide" includes derivatives thereof, such as backbone modifications, e.g., phosphorothioate derivatives, employed to stabilize the oligonucleotide. All such modifications are contemplated equivalents of the antisense

oligonucleotides of the invention. The following discussion provides examples of the kinds of modifications that may be employed, but those of skill in the art will readily recognize others. Non-naturally occurring backbones carrying bases and capable of base pairing to natural nucleic acids both known and not as yet invented, may be substituted for DNA or RNA oligonucleotides; such backbones may prove more stable than DNA or RNA. For example, the antisense oligonucleotides may be provided in stabilized form, e.g. with phosphotriester linkages, or by blocking against exonuclease attack with methylphosphonodiester linkages, with 3' deoxythymidine, as a phenylisourea derivative, or by linking other molecules such as aminoacridine or polylysine to the 3' end of the oligonucleotide. See e.g., Anticancer Research 10: 1169-1182, at 1171-2 (1990), the teaching of which is incorporated herein by reference. Though exemplified herein by single-stranded DNA molecules, it will be recognized that non-DNA backbones may be substituted. For instance, an RNA or RNA-DNA hetero-oligomer antisense molecule would be useful if one desired the antisense sequence be less stable or more tightly binding than a DNA oligonucleotide. Base analogues may be substituted for the commonly found A (adenosine or deoxyadenosine), G (guanosine or deoxyguanosine), C (cytidine or deoxycytidine), T (thymine) or U (uridine). Examples include, but are not limited to, 7-aza-G and 5-methyl-C. Such base analogues are useful for adjusting Tm of an oligonucleotide or a segment thereof. Tm, or melting temperature, is a measure of binding between two strands of a double-stranded nucleic acid. Substitution of rT (ribothymidine) for U or dU (deoxyuridine) for T are also possible. Other strategies include attaching oligonucleotides to DNA-protein complexes or cationic liposomes as exemplified in the Examples following. For antisense oligonucleotides supplied exogenously, increased selectivity for cell type may be achieved by linking antisense oligonucleotide complexes of the invention to natural ligands of the target cell or cell-specific antibodies, or to synthetic ligands that will bind to the target cell. The oligonucleotide may also be at least partially double stranded, either by binding to a distinct oligonucleotide or by formation of a hairpin, either at one or both termini or internally as long as the oligonucleotide is still able to decrease expression of the desired gene in a cell.

8 The present invention is not limited to any particular method of making the antisense oligonucleotides. The antisense oligonucleotides may be produced by any method known to the art. While those exemplified herein were synthesized using an automated synthesizer, expressed nucleotides made by an expression vector used for gene therapy, such as an adenoviral, retroviral, or plasmid vector can be designed to produce antisense RNA when introduced into a cell. Use of other synthetic chemistries is possible, see for example, Ulhmann and Peyman (1990) Chemical Rev. 90: 544-584. Other methods of making these oligonucleotides will be evident to those with skill in the art. It will be recognized by those in the art that having shown that the invention is operative with the exemplified oligonucleotides and in accordance with other teachings of the present invention, those of ordinary skill in the art are enabled to design and test oligonucleotides not exemplified herein, that are also operative.

9 In another embodiment, the present invention relates to compounds for use in the treatment or diagnosis of disease. The compounds of the present invention are antisense oligonucleotides as described above, able to reduce the expression of the gene they target, specifically, raf-1, Ha-ras or HER-2. The compounds of the present invention can be used as therapeutic agents to treat or diagnose disorders or diseases related to the expression of raf-1, Ha-ras or HER-2. Such diseases include but are not limited to, cancer, restenosis, osteoarthritis, neurological and intestinal abnormalities, pre-eclampsia, among others.

10 The compounds of the present invention may be used to detect the level or presence of raf-1, Ha-ras or HER-2 RNA or DNA in a sample, said sample being a cell, cell extract, purified DNA or RNA from cells, tissue, or organ, or sections of tissues or organs, or diagnose an increase in raf-1, Ha-ras or HER-2 RNA in a cell, by detecting raf-1, Ha-ras or HER-2 RNA. The level of raf-1, Ha-ras or HER-2 RNA can be detected by extracting cellular RNA and

detecting the level of raf-1, Ha-ras or HER-2 RNA using a hybridization assay, such as a Northern hybridization assay wherein the antisense oligonucleotides are labeled with a detectable label, or alternatively, by in situ assay of a cell or organ or tissue section using in situ hybridization techniques known to persons in the art. In addition, the compounds of the present invention can be used in a polymerase chain reaction assay as primers for the detection of raf-1, Ha-ras or HER-2 RNA or DNA in cells by methods well known in the art. The compounds of the present invention may be labeled using any of a variety of labels and method of labeling. Examples of types of labels which can be used in the present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, fluorescent labels, and chemiluminescent labels. Such assays may also be useful for in vitro testing of potential drugs for treating a disease involving raf-1, Ha-ras or HER-2 RNA, such as cancer, or to monitor the effect of the drug on raf-1, Ha-ras or HER-2 RNA expression. Cell lines useful for in vitro drug testing would be those expressing raf-1, Ha-ras or HER-2 RNA. For instance, cells expressing Ras include, but are not limited to,T24, Hs578T, SK-CO-1, Calu1 among others [Bos, J. L. (1988) Mutation Research 195: 255-271]; Cells expressing Raf-1 include, but are not limited to, SQ-20B, JSQ-3 and other SCCHN cell lines, among others [Weichselbaum, R. R. et al. (1988) Int. J. Radiation Oncology Biol. Phys. 15: 575-579; Weichselbaum, R. R. et al. (1986) PNAS USA 83: 2684-2688]. Cells expressing HER-2 include, but are not limited to SK-OV-3, ZR-75-1, MDA-MB-435, and MDA-MB-453, among others.

11 As was discussed above, a signal transduction pathway, with raf-1, Ha-ras or HER-2 as central elements, leads to cellular drug resistance and radioresistance. Chemotherapy and radiation are two major forms of adjuvant therapy for various types of cancer. The ability to revert drug/radiation resistant tumor cells thereby rendering them drug/radio-sensitive and vulnerable to drug and radiation treatments, provides a valuable method in the treatment of tumors. The antisense oligonucleotide compositions of the present invention are able to ameliorate or revert the drug resistance and radioresistance of tumor cells. Raf-1 expressing tumors include stomach, and squamous cell carcinoma of the head and neck (upper aero-digestive track); ras expressing tumors include bladder, breast, lung, colon, pancreas, prostate; HER-2 expressing tumors include breast, ovarian, cervical, lung, prostate, head and neck cancers.

12 The oligonucleotides can be used in a method for treating diseases or conditions involving raf-1, Ha-ras or HER-2 expression, or where the modulation of these genes is desired. The method would include administering an effective amount of one or more of the compounds of the present invention, or one or more raf-1, Ha-ras or HER-2 antisense oligonucleotide(s) to a patient requiring such a treatment, such that the level of the targeted RNA or protein is decreased. The antisense oligonucleotides can be prepared for administration by methods known in the art which can include filtering to sterilize the solution of antisense oligonucleotides, diluting or concentrating the solution, adding a stabilizer to the solution, lyophilizing the solution to produce the oligonucleotides in dried form for ease in transportation and storage. Improvement of oligonucleotide uptake has been achieved with different systems of vectorization including liposomes (neutral, cationic, immunoliposome), nanoparticles, or covalent attachment of a carrier. Advantageously, the antisense oligonucleotides are combined with "sterically stabilized" liposomes (S-liposomes) which have been developed and are suitable for therapeutic applications such as sustained drug release and selective delivery of drugs to specific targets [Reviewed in Allen, T. M. (1994) TIPS 15, 215-220 and Gregoriadis, G. and Florence, A. T. (1993) Drugs 45: 15-28]. Long circulating half-lives and the ability of the S-liposomes to localize in high concentration in solid tumors make them useful in cancer treatment. The liposome-antisense delivery can be passive for example by simple fusion with the cell, or active by attachment of antibodies or other proteins to the liposome surface to cause specific targeting [Allen, T. M. et al. (1994) J. Lipos. Res. 4: 1-25; Mori, A. et al. (1991) FEBS Lett. 284: 263-266]. Further, the antisense oligonucleotide treatment solution can be in the form of a mixed solution which contains the antisense oligonucleotides described above and at least one other antigen or

oligonucleotide, as long as the added compound does not interfere with the effectiveness of the antisense oligonucleotide treatment and adverse reactions such as toxicity are not increased additively or synergistically.

13 The antisense oligonucleotide treatment solution may be stored in a sealed vial, ampule or the like. The present treatment can be administered in the form of a spray for intranasal administration, or by nose drops, inhalants, eye drops, skin patch, suppository, mini-pump implant, or a capsule, liquid suspension or elixirs formulated for oral administration. In the case where the treatment is in dried form, the treatment can be dissolved or suspended in sterilized distilled water or saline before administration. Any inert carrier is preferably used, such as saline, phosphate buffered saline, or any such carrier in which the antisense oligonucleotides have suitable solubility.

14 Generally, the method of administration of treatment may depend on the organ or organs targeted. The compounds or treatment may be administered orally, subcutaneously, intravenously, or intramuscularly or intracranially by direct injection. For example, in the lung, the composition would be administered as an inhalant, or intravenously; in the breast, head or neck, intravenously or direct injection; in the bladder, ovaries, or pancreas, intravenously. These methods of administration are known to people in the art.

15 The compounds of the present invention can be administered in a dose effective for the production of a decrease in raf-1, Ha-ras or HER-2 and resulting in an improvement of the patient's disease, or amelioration of the patient's disease symptoms. The treatment may be in the form of a single dose or in multi-dose program. At low (sub-optimal) concentrations of the oligonucleotides, the effect may be additive or even synergistic. When providing a patient with antisense oligonucleotides, the dosage administered will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of 1 pg/kg to 500 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

16 Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in this art which are obvious to those skilled in the art are within the spirit and scope of the present invention.

17 The following MATERIALS AND METHODS were used in the examples that follow.

18 Oligonucleotides

19 Phosphorothioated ASO directed at or near the initiation codon were synthesized by Midland Certified Reagent Co., Midland, Tex. for the raf-1 (5'-TCCCTGTATGTGCTCCAT-3')(SEQ ID NO:1), Ha-ras (5'-TATTCCGTCAT-3') (SEQ ID NO:2), and HER-2 (5'-TCCATGGTGCTCACT-3') (SEQ ID NO:3) genes.

20 Two controls for each gene, either a sense and a scrambled (for raf-1) or two different scrambled (for Ha-ras and HER-2) oligonucleotides were also synthesized. The scrambled oligomers have the same base composition as antisense but in a different, random order. The sequences for the raf-1 controls are 5'-ATGGAGCACATACAGGGA-3' (sense) (SEQ ID NO:4) and 5'-CTAGCCTATCTGTCTTCG-3' (scrambled) (SEQ ID NO:5); for Ha-ras 5'-TTATACGTCCT-3' (scrambled 1) (SEQ ID NO:6) and 5'-TTATACGTCCT-3' (scrambled 2) (SEQ ID NO:7); and for HER-2 5'-CACTGGTTGCACCTT-3' (scrambled 1) (SEQ ID NO:8) and 5'-CTAGCCATGCTTGTC-3' (scrambled 2) (SEQ ID NO:9).

21 Cell Culture and Treatment

22 Squamous cell carcinoma of the head and neck (SCCHN) cell lines JSQ-3 (Weichselbaum R. R., et al. (1988) Int. J. Radiation Oncology Biol. Phys. 15:575-579), SQ-20B (Weichselbaum R. R., et al. (1986) Proc. Natl. Acad. Sci.

USA 83:2684-2688) and SCC-61 (Weichselbaum R. R., et al. (1986) Proc. Nail. Acad. Sci. USA 83:2684-2688), which were kind gifts from Dr. Ralph Weichselbaum, University of Chicago, were maintained in Minimum Essential Medium with Earle's salts (EMEM), supplemented with 10% heat inactivated fetal bovine serum; 50 .mu.g/ml each of penicillin, streptomycin and neomycin; 2 mM L-glutamine; 0.1 mM non-essential amino acids, 1 mM pyruvate and 0.4 .mu.g/ml hydrocortisone. Human ovarian (SK-OV-3) and bladder (T24) carcinoma cell lines (obtained from ATCC, Rockville, Md.) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 50 .mu.g/ml each of penicillin, streptomycin and neomycin and 2 mM L-glutamine. Normal human non-tumor breast cell line MCF 10A (ATCC, Rockville, Md.) was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 5% horse serum, 20 ng/ml epithelial growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 50 .mu.g/ml each of penicillin, streptomycin and neomycin and 2 mM L-glutamine.

23 For oligonucleotide treatment, the cells were plated at 1.times.10.sup.5 cells/well in 6-well tissue culture plates. Twenty-four hours later, at approximately 40-60% confluency, the cells were transfected with the oligonucleotides, facilitated by Lipofectin Reagent, using essentially the protocol supplied by the manufacturer, Life Technologies, Inc. After 6 hours, the lipofection solution was removed and the monolayer washed with fresh medium containing 8 mM L-glutamine and 20% serum. The cells were then incubated for an additional 16-18 hours in 1 ml of this medium.

24 Radiobiology

25 Cellular response to radiation was evaluated by the colony survival assay. Exponentially growing monolayer cultures of each cell line were treated with the oligonucleotides as described above. The cells were harvested 24-48 hours later, suspended in fresh medium and irradiated at room temperature with graded doses of .sup.137 Cs .gamma. rays at a dose of approximately 36 Gy/minute in a J. L. Shepard and Associates Mark I irradiator. Afterward, the cells were diluted and plated at a concentration of 300 to 5000 cells per well in a 6-well tissue culture plate. Two to three days after plating, the cells were supplemented with 0.5 ml of serum plus 5 .mu.g/ml hydrocortisone. Approximately 7-14 days later, the cells were stained with 1% crystal violet and colonies (comprising 50 or more cells of normal appearance) were scored. Survival curves were plotted as the log of the survival fraction versus the radiation dose using Sigma-Plot Graphics program. D.sub.10 (the dose required to reduce survival to 10%) values were calculated from the initial survival data.

26 Protein Analysis

27 After oligonucleotide treatment, cells for protein analysis were trypsinized, pelleted, rinsed with PBS and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 .mu.g/ml aprotinin and 1 mM sodium orthovanidate in PBS) (Santa Cruz Biotechnology, Inc). After shearing with a 26 gauge needle, 100 .mu.g/ml Phenylmethylsulfonyl fluoride (PMSF) was added, the lysate incubated on ice for 30-60 minutes and centrifuged at 13,000.times.g for 20 minutes at 4.degree. C. to pellet insoluble material. Protein concentration was determined using the micro-BCA Protein Assay Kit (Pierce Biochemicals).

28 Protein lysate (40 .mu.g for Ha-ras, 5 .mu.g for raf-1 and HER-2) was mixed with an equal volume of 2.times. protein sample buffer (0.05 M Tris (pH 6.8), 3% SDS, 20% Glycerol, 6% 2-Mercaptoethanol and 0.001% Bromophenol blue) boiled for 5 minutes, loaded on a 12% (5% stacking gel) SDS/Polyacrylamide gel and electrophoresed at 200V for 8 hours. The protein was transferred to nitrocellulose membrane as previously described [Janat, M. F. et al. (1994) Mol. Cell. Diff. 2:241-253]. Preparation of membrane and incubation with the primary and secondary antibodies was performed essentially as described in a protocol supplied by Santa Cruz Biotechnology, Inc., with the exception that incubation with the primary antibody was extended to 1 hour for raf-1, 2 hours for HER-2 and 4 hours for Ha-ras and wash times of 15 minutes per wash were used. The primary antibodies for HER-2 (neu C-18) and Ha-ras (ras C-20) were

obtained from Santa Cruz Biotechnology, Inc. The anti-raf-1 antibody was a kind gift from Dr. Andrew Laudana, University of Vermont [McGrew, B. R. et al. (1992) Oncogene 7: 33-42]. The washings after addition of the secondary antibody (Anti-mouse IgG-HRP, Santa Cruz Biotechnology, Inc.) were also lengthened to 15 minutes per wash.

29 Visualization of the protein was accomplished using the ECL Chemo-luminescent Western Blotting Kit (Amersham) according to the manufacturer's protocol.

30 EXAMPLE 1

31 Human tumor cell lines JSQ-3 and SQ-20B, which display a high level of radiation resistance, were established from SCCHN tumors which failed radiotherapy [Weichselbaum, R. R. et al. (1988) supra; Weichselbaum, R. R. (1986) supra]. An activated form of the raf-1 oncogene was isolated from these cell lines via the NIH 3T3 transfection assay [Kasid, U. et al. (1987) Science 237: 1039-1041]. These and other studies with radioresistant non-cancerous skin fibroblast cell lines from a cancer-prone family [Chang, E. H. et al. (1987) Science 237: 1036-1039; Pirollo, K. F. et al. (1989) Int. J. Radiat. Biol. 55: 783-796] have clearly linked activation of raf-1 to increased RR. We, therefore, wished to determine if treatment of these cell lines with anti-raf-1 ASO would inhibit raf-1 expression and revert this phenotype. Consequently, JSQ-3 and SQ-20B were treated with increasing concentrations of anti-raf-1 oliogonucleotides and the level of the raf-1 protein expression determined. Since we have previously shown that lipofectin enhanced uptake of these compounds resulting in lower effective doses, a commercially available liposome preparation (Lipofectin) was used in these and all subsequent experiments to facilitate delivery of the oligonucleotides. As shown in FIG. 1A, Raf-1 protein expression in JSQ-3 cells is completely inhibited by treatment with 1 .mu.M of antisense raf-1, with significant inhibition evident at a concentration as low as 0.1 .mu.M. The specificity of the inhibition was demonstrated by treating the cells with Lipofectin alone (0) or with a raf-1 sense oligonucleotide (S). No decrease in protein expression as compared to the untreated control cells (C) was observed in either case. A similar pattern of results was observed with cell line SQ-20B. However, with this cell line, a 3 .mu.M concentration was necessary to effect complete inhibition of raf-1 protein, with only approximately 50% inhibition observed at 1 .mu.M. Also shown in FIG. 1A is the effect of raf-1 ASO on a radiosensitive SCCHN cell line, SCC61. Here also treatment with raf-1 ASO was able to specifically inhibit raf-1 protein expression.

32 EXAMPLE 2

33 We next examined the effect of anti-raf-1 on the RR level of these cells. FIG. 1B demonstrates a dramatic increase in radiosensitivity for both cell lines after ASO treatment. This response, particularly in the JSQ-3 cells, is dose dependent. The D.sub.10 value for JSQ-3 drops from the highly resistant level of 6.3.+-.0.16 Gy to 4.9.+-.0.05 Gy, a value much closer to the level considered to be radiosensitive, after treatment with 1 .mu.M raf-1 ASO. Even a dose as low as 0.3 .mu.M is capable of significantly sensitizing these cells to killing by .gamma.-radiation. Similarly, the resistance level of SQ-20B is reduced from 6.8.+-.0.31 Gy to 5.1.+-.0.09 Gy. This change of approximately 1.5 Gy was found to be highly statistically significant ($p<0.001$). Here also, the specificity of the oligonucleotide is evident since treatment with either Lipofectin (Liposome) alone, a sense, or a scrambled oligomer had minimal or no effect on the RR level of the cells. Moreover, the differences between JSQ-3 and SQ-20B with respect to their level of sensitization after ASO treatment correlates with that observed in the protein analysis, indicating that this decrease in radioresistance is directly related to Raf-1 expression. An example of the survival curves produced in these experiments is given as FIG. 2A.

34 By contrast, treatment of SCC61 cells, which are highly radiosensitive, with 1 .mu.M of raf-1 ASO had no significant effect on their radiation response level. The D.sub.10 value of the control and sense treated cells was found to be 3.3.+-.0.4 Gy and 3.4.+-.0.06 Gy, respectively, while that of the ASO treated

was 3.0.+-.0.4 Gy indicating a slight, but not significant, sensitization of the cells by the raf-1 ASO.

35 EXAMPLE 3

36 Our previous studies have placed raf-1 in a central role in a proposed signal transduction pathway leading to cellular RR. If this hypothesis is correct then treatment with raf-1 ASO of cells which have activated or abnormally expressed genes upstream of raf-1 in this pathway should block signaling leading to decreased radioresistance. Therefore, we transfected human tumor cell lines SK-OV-3 and T24 with raf-1 ASO. These two cell lines possess either elevated levels of HER-2 [Chan, S. D. et al. (1995) J. Biol. Chem. 270: 22608-22613] or a mutated Ha-ras genes [Tabin, C. J. et al. (1982) Nature 300: 143-149], respectively. Both of these genes have been placed upstream of raf-1 in the signal transduction pathway [Daum, G. et al. (1994) TIBS 19: 474-480; Rapp, U. R. et al. (1988) The Oncogene Handbook (T. curran, J. E. P. Reddy, and A. Skala, Eds.) pp. 213-252, Elsevier, Amsterdam]. The presence of 1 .mu.M antisense raf-1 was able to significantly reduce the RR level of both cell lines from 6.83.+-.0.42 Gy to 4.90.+-.0.08 Gy for SK-OV-3 and from 5.93.+-.0.36 Gy to 3.58.+-.0.36 Gy for T24 (FIG. 3). As before, treatment with either the sense or scrambled oligo did not decrease on the RR level of the cells. This sensitization is also evidenced by the differences in the survival curves between control SK-OV-3 cells, and those treated with 1 uM raf-1 antisense oligonucleotides (FIG. 2B). As a control, a normal radiosensitive breast epithelial cell line, MCF10A, was also used. As with SCC61, no effect on the D.sub.10 values was observed with this cell line, again indicating no further sensitization of the cells at this concentration of ASO.

37 EXAMPLE 4

38 To further confirm the role of these activated oncogenes in signal transduction and the RR phenotype, SK-OV-3, T24 and MCF10A cells were also treated with ASO against the Ha-ras gene. Since, as a growth factor receptor, HER-2 is upstream of ras in the proposed signalling pathway, it would be expected that the Ha-ras ASO would affect both the HER-2 expressing cells (SK-OV-3), and the cells containing mutant Ha-ras (T24). FIG. 4 shows this to be the case. D.sub.10 for the 3 .mu.M ASO treated T24 cells is decreased from the control value of 5.93.+-.0.36 Gy to the significantly more radiosensitive value of 4.29.+-.0.20 Gy, while that for SK-OV-3 is lowered from 6.83.+-.0.42 Gy (Control) to 5.47.+-.0.03 Gy after introduction of the anti-ras molecule. As before, there was no significant decrease in radiation survival in the control MCF10A cells after ASO treatment.

39 EXAMPLE 5

40 These same three cell lines were also treated with antisense oligonucleotides directed against HER-2. Over 80% inhibition of HER-2 protein was observed in the SK-OV-3 cells with HER-2 ASO at a concentration as low as 0.3 .mu.M (FIG. 5A). However, significant HER-2 protein inhibition in the T24 cells is found only at 3 .mu.M and none is evident in MCF10A, even at this relatively high concentration of ASO. The effect of HER-2 ASO on the RR level of these cells was also examined (FIG. 5B). While treatment with 0.1 and 0.3 .mu.M HER-2 ASO had some effect on the radiosensitivity of the SK-OV-3 cells, treatment with 1 .mu.M HER-2 ASO significantly sensitized the SK-OV-3 cells, reducing the D.sub.10 value from 6.83.+-.0.42 Gy to 4.88.+-.0.43 Gy, a result virtually identical to that observed after treatment of SK-OV-3 cells with 1 .mu.M anti-raf-1 ASO. This change of approximately 2 Gy is highly statistically significant ($p<0.001$) and represents a 5 fold increase in sensitivity to radiation killing these cells. Surprisingly, the radiosensitivity of the T24 cells was also altered by treatment with 1 .mu.M HER-2 ASO.

41 Discussion

42 In our previous studies we examined the relationship between activation of oncogenes and the phenomenon of cellular radiation resistance [Pirollo, K. F.

et al. (1993) Rad. Res. 135: 234-243; Pirollo, K. F. et al. (1989), supra]. We proposed, based upon our findings and those of other researchers, the presence of a signal transduction pathway, analogous to that for cell growth and differentiation, leading to radiation resistance to killing by ionizing radiation [Pirollo, K. F. et al. (1993), supra]. In the studies described above, we present evidence confirming such a pathway. Activation of the raf-1 gene has been shown to be related to radiation resistance in SCCHN and in the non-cancerous skin fibroblasts from a cancer-prone family with Li-Fraumeni syndrome [Kasid, U. et al (1987), supra; Chang, E. H. et al. (1987), supra; Pirollo, K. F. et al. (1989), supra; Kasid, U. et al. (1989) Cancer Res. 49: 3396-3400]. Raf-1 is also known to play a central part in signal transduction via the MAP Kinase pathway [Campbell, J. S. et al. (1995) Recent Progress in Hormone Research 50: 131-159; Daum, G. et al. (1994), supra]. In this Ras/Raf/MEK/ERK pathway, a small guanine nucleotide-binding protein links receptor tyrosine kinase activation to a cytosolic protein kinase cascade [Marshall, C. J. (1995) Cell 80: 179-185]. The protein-protein interaction between Ras and Raf, through the CRI region on Raf-1 and the effector site of Ras, leads to a partial activation of Raf-1. Full activation of Raf-1 is achieved by another tyrosine kinase generated signal [Marshall, C. J. (1995), supra; Fabian, J. R. et al. (1994) PNAS USA 91: 5982-5986] and leads to the phosphorylation and activation of MEK, its only known physiological substrate. This in turn results in the activation of ERK1 and/or ERK2. The substrates for the ERKs in the nucleus are transcription factors, activation of which can set in motion a wide range of events. Raf-1 has also been shown to be a key component in the mammalian response to damage by ultraviolet light [Devary, Y. et al. (1992) Cell 41:1081-1091; Radler-Pohl, A. et al. (1993) EMBO J. 12: 1005-1012]. This "U.V. response" has been proposed to have a protective function, in a manner analogous to that of the bacterial "SOS" system. It was shown by Devary et al. that this pathway originates at the cell membrane and includes activation of Src, and Ha-Ras as well as Raf-1 in a signaling cascade leading to activation of transcription factor AP-1 and nuclear factor kappa B [Devary, Y. (1992), supra].

43 Protooncogenes and their oncogenic counterparts such as HER-2 (a homologue to an epidermal growth factor receptor) and ras are known to be upstream of raf-1 in the Map Kinase pathway [Daum, G. (1994), supra; Rapp, U. R. et al. (1988),supra]. The ability, as demonstrated here, of antisense oligonucleotides directed against raf-1 to revert the RR phenotype of cells containing activated ras or overexpressing HER-2 is clear evidence of signaling through raf-1 leading to RR. This is further supported by the ability of antisense ras oligomers to sensitize HER-2 overexpressing SK-OV-3 cells to .gamma.-radiation killing. Although HER-2 is upstream of ras in the signal transduction pathway, ASO directed against HER-2 was also able to affect the RR level of ras transformed T24 cells. These findings may be explained in part by the established interaction between the EGF receptor and adaptor protein/guanine nucleotide exchange factor (Grb2/Sos). Buday and Downward have shown that EGF-induced activation of nucleotide exchange on p21.sup.ras proceeds through recruitment of Sos to a complex with the EGF receptor and Grb2 at the plasma membrane and that inhibition of this Grb2-EGFR interaction can inhibit activation of ras [Buday, L. and Downward, J. (1993) Cell 73: 611-620]. Therefore, it is conceivable that inhibition of the HER-2 protein by ASO can disrupt this interaction, and thus p21ras nucleotide exchange, and interfere with signalling through ras resulting in decreased RR.

44 Further support for the existence of the pathway leading to RR is found in the work of Morrison, et al. [1988, PNAS USA 85:8855-8859] and Haimovitz-Friedman, et al. (1991, Can. Res. 51: 2552-2558). These investigators found that bFGF, synthesis of which is stimulated in epithelial cells by .gamma.-irradiation [Haimovitz-Friedman et al. (1991), supra] and which in turn activates Raf-1 protein kinase [Buday and Downward (1993), supra], can protect against radiation-induced cell killing [Haimovitz-Friedman et al. (1991), supra]. Our hypothesis of a signal transduction pathway is further confirmed in a recent report by Kasid et al. which showed that Raf-1 is phosphorylated/activated after exposure to ionizing radiation by upstream protein tyrosine kinases [Kasid, U. et al. (1996) Nature 382: 813-816].

45 These studies, supporting a pathway, with raf-1 as a central element, leading to cellular radioresistance are also clinically significant in a number of ways. Radiation is one of the major forms of adjuvant therapy for various types of cancer. Understanding the molecular mechanisms leading to the failure of a significant fraction of tumors to respond to radiotherapy opens the door to the development of new methods of intervention to radio-sensitize tumors, resulting in more effective cancer treatments. In this vein, our use of antisense oligonucleotides to radiosensitize human tumor cells not only establishes the signal transduction pathway, but also demonstrates the potential of these molecules as cancer therapeutic agents showing that ASO directed against a focal point in the pathway can be effective in a number of different tumor types. In a similar way, using mouse m5S cells, Taki et al. [Taki, T. et al. (1996) Bio. Biop. R. 223:834-438] also recently found that ASO against RAD51, a gene involved in recombination and DNA repair, could increase radiosensitivity.

46 The use of liposome facilitated delivery of the ASO permits significantly lower effective concentrations of oligomers to be used, a step towards eliminating one of the major drawbacks to the clinical use of antisense therapy. The efficacy of ASO is also advantageous for clinical use. At the concentrations employed in these studies, none of the ASOs increased the sensitivity of control radiosensitive cell lines SCC61 and MCF10A, thereby demonstrating that the use of ASO to ameliorate radioresistance is not deleterious to normal tissues thereby strengthening the potential usefulness of ASO in cancer treatment.

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- - (ii) MOLECULE TYPE: DNA
- - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
- - CTAGCCATGC TTGTC - # - #
- # 15

CLAIMS:

What is claimed is:

1. A method for reducing radiation or drug resistance of a cell, in vitro,

which does not overexpress HER-2, said method comprising introducing into said cell an antisense nucleic acid comprising a segment complementary to HER-2 in an amount effective to reduce said radiation or drug resistance.

2. The method of claim 1 wherein said cell is a carcinoma cell selected from the group consisting of breast, bladder, prostate, head, neck, lung, colon, pancreas, cervical, ovarian, and stomach carcinoma cells.

3. The method of claim 1 wherein said antisense nucleic acid is introduced by association with a liposome.

4. The method of claim 1 wherein said antisense nucleic acid comprises SEQ ID NO:3.

5. A method for reducing radiation or drug resistance of a cell, in vitro, which does not overexpress raf-1, said method comprising introducing into said cell an antisense nucleic acid comprising a segment complementary to raf-1 in an amount effective to reduce said radiation or drug resistance.

6. The method of claim 5 wherein said cell is a carcinoma cell selected from the group consisting of breast, bladder, prostate, head, neck, lung, colon, pancreas, cervical, ovarian, and stomach carcinoma cells.

7. The method of claim 5 wherein said antisense nucleic acid is introduced by association with a liposome.

8. The method of claim 5 wherein said antisense nucleic acid comprises SEQ ID NO:1.

9. A method for reducing radiation or drug resistance of a cell, in vitro, which does not comprise a mutant Ha-ras, said method comprising introducing into said cell an antisense nucleic acid comprising a segment complementary to Ha-ras in an amount effective to reduce said radiation or drug resistance.

10. The method of claim 9 wherein said cell is a carcinoma cell selected from the group consisting of breast, bladder, prostate, head, neck, lung, colon, pancreas, cervical, ovarian, and stomach carcinoma cells.

11. The method of claim 9 wherein said antisense nucleic acid is introduced by association with a liposome.

12. The method of claim 9 wherein said antisense nucleic acid comprises SEQ ID NO:2.

WEST

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Main Menu | Search Form | Posting Counts | Show S Numbers | Edit S Numbers | Preferences | Cases

Search Results -

Term	Documents
HER-2.USPT.	243
HER-2S	0
ANTISENS$	0
ANTISENS.USPT.	20
ANTISENSC.USPT.	7
ANTISENSE.USPT.	13874
ANTISENSED.USPT.	7
ANTISENSEE.USPT.	1
ANTISENSEEARLY.USPT.	1
ANTISENSEHES.USPT.	1
ANTISENSEION:.USPT.	1
(HER-2 AND (ANTISENS$ OR RIBOYZME$)).USPT.	100

There are more results than shown above. Click here to view the entire set.

Database: US Patents Full-Text Database
US Pre-Grant Publication Full-Text Database
JPO Abstracts Database
EPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Search: L1

Refine Search

Recall Text | Clear

Search History

DATE: Wednesday, December 18, 2002 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
	DB=USPT; PLUR=YES; OP=ADJ		
L1	her-2 and (antisens$ or riboyzme$)	100	L1

END OF SEARCH HISTORY

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Search Results -

Term	Documents
HER-2.DWPI,EPAB,JPAB.	34
HER-2S	0
ANTISENS$	0
ANTISENS.DWPI,EPAB,JPAB.	6
ANTISENSE.DWPI,EPAB,JPAB.	5887
ANTISENSENCE.DWPI,EPAB,JPAB.	1
ANTISENSENUCLEOTIDE.DWPI,EPAB,JPAB.	1
ANTISENSEOR.DWPI,EPAB,JPAB.	1
ANTISENSEPRIMER.DWPI,EPAB,JPAB.	1
ANTISENSES.DWPI,EPAB,JPAB.	1
ANTISENSE-ACETATE.DWPI,EPAB,JPAB.	1
(HER-2 AND (ANTISENS$ OR RIBOYZME$)).JPAB,EPAB,DWPI.	4

There are more results than shown above. Click here to view the entire set.

Database: US Patents Full-Text Database; US Pre-Grant Publication Full-Text Database; JPO Abstracts Database; EPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

Search: L3

Refine Search

Recall Text | Clear

Search History

DATE: Wednesday, December 18, 2002 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L3	her-2 and (antisens$ or riboyzme$)	4	L3
L2	L1	0	L2
DB=USPT; PLUR=YES; OP=ADJ			
L1	her-2 and (antisens$ or riboyzme$)	100	L1

END OF SEARCH HISTORY

WEST

Generate Collection | Print

Search Results - Record(s) 1 through 4 of 4 returned.

☐ 1. Document ID: FR 2807437 A1 WO 200177323 A1

L3: Entry 1 of 4 File: DWPI Oct 12, 2001

DERWENT-ACC-NO: 2001-658141
DERWENT-WEEK: 200176

TITLE: New polypeptide, erbin, useful e.g. for diagnosis and treatment of tumors, interacts with the intracellular part of the HER-2 receptor

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: WO 200011013 A1 AU 9955762 A

L3: Entry 2 of 4 File: DWPI Mar 2, 2000

DERWENT-ACC-NO: 2000-246530
DERWENT-WEEK: 200021

TITLE: Modified nucleomonomers, used in physiologically stable, non-toxic oligomers used to inhibit expression of nucleic acids and in gene regulation, antisense technology and diagnostics

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Clip Img | Image

☐ 3. Document ID: US 6027892 A

L3: Entry 3 of 4 File: DWPI Feb 22, 2000

DERWENT-ACC-NO: 2000-194828
DERWENT-WEEK: 200017

TITLE: Reducing radiation or drug resistance in a cell comprises introduction of antisense nucleic acid for treating or diagnosing cancer, restenosis, osteoarthritis, neurological and intestinal abnormalities and pre-eclampsia

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

☐ 4. Document ID: WO 9948906 A1 AU 9933642 A US 5968748 A

L3: Entry 4 of 4 File: DWPI Sep 30, 1999

DERWENT-ACC-NO: 1999-610749
DERWENT-WEEK: 200009

TITLE: New antisense sequences used to treat hyperproliferative conditions, especially cancer

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

Generate Collection | Print

Term	Documents
HER-2.DWPI,EPAB,JPAB.	34
HER-2S	0
ANTISENS$	0
ANTISENS.DWPI,EPAB,JPAB.	6
ANTISENSE.DWPI,EPAB,JPAB.	5887
ANTISENSENCE.DWPI,EPAB,JPAB.	1
ANTISENSENUCLEOTIDE.DWPI,EPAB,JPAB.	1
ANTISENSEOR.DWPI,EPAB,JPAB.	1
ANTISENSEPRIMER.DWPI,EPAB,JPAB.	1
ANTISENSES.DWPI,EPAB,JPAB.	1
(HER-2 AND (ANTISENS$ OR RIBOYZME$)).JPAB,EPAB,DWPI.	4

There are more results than shown above. Click here to view the entire set.

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Search Results -

Term	Documents
HER-2.PGPB.	119
HER-2S	0
ANTISENS$	0
ANTISENS.PGPB.	5
ANTISENSC.PGPB.	2
ANTISENSE.PGPB.	5253
ANTISENSEAND.PGPB.	2
ANTISENSED.PGPB.	17
ANTISENSEDEOXYOLIGONUCLEOTIDE.PGPB.	1
ANTISENSELNNER.PGPB.	1
ANTISENSENESTED.PGPB.	1
(HER-2 AND (ANTISENS$ OR RIBOYZME$)).PGPB.	42

There are more results than shown above. Click here to view the entire set.

Database: US Patents Full-Text Database; US Pre-Grant Publication Full-Text Database; JPO Abstracts Database; EPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

Search: L4

Refine Search

Recall Text | Clear

Search History

DATE: Wednesday, December 18, 2002 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=PGPB; PLUR=YES; OP=ADJ			
L4	her-2 and (antisens$ or riboyzme$)	42	L4
DB=JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L3	her-2 and (antisens$ or riboyzme$)	4	L3
L2	L1	0	L2
DB=USPT; PLUR=YES; OP=ADJ			
L1	her-2 and (antisens$ or riboyzme$)	100	L1

END OF SEARCH HISTORY

WEST

Generate Collection | Print

Search Results - Record(s) 1 through 10 of 62 returned.

☐ 1. Document ID: US 6495553 B1

L8: Entry 1 of 62 File: USPT Dec 17, 2002

US-PAT-NO: 6495553
DOCUMENT-IDENTIFIER: US 6495553 B1

TITLE: Methods and compositions for overcoming resistance to biologic and chemotherapy

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 2. Document ID: US 6472204 B1

L8: Entry 2 of 62 File: USPT Oct 29, 2002

US-PAT-NO: 6472204
DOCUMENT-IDENTIFIER: US 6472204 B1

TITLE: Methods for retroviral mediated gene transfer employing molecules, or mixtures thereof, containing retroviral binding domains and target cell binding domains

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 3. Document ID: US 6471968 B1

L8: Entry 3 of 62 File: USPT Oct 29, 2002

US-PAT-NO: 6471968
DOCUMENT-IDENTIFIER: US 6471968 B1

TITLE: Multifunctional nanodevice platform

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 4. Document ID: US 6451312 B1

L8: Entry 4 of 62 File: USPT Sep 17, 2002

US-PAT-NO: 6451312

DOCUMENT-IDENTIFIER: US 6451312 B1

TITLE: VEGF-gelonin for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 5. Document ID: US 6426042 B1

L8: Entry 5 of 62 File: USPT Jul 30, 2002

US-PAT-NO: 6426042
DOCUMENT-IDENTIFIER: US 6426042 B1

TITLE: Methods and kits for improving retroviral-mediated gene transfer utilizing molecules, or mixture thereof, containing retroviral binding domains and target cell binding domains

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 6. Document ID: US 6410313 B1

L8: Entry 6 of 62 File: USPT Jun 25, 2002

US-PAT-NO: 6410313
DOCUMENT-IDENTIFIER: US 6410313 B1

TITLE: Gene delivery system and methods of use

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 7. Document ID: US 6410271 B1

L8: Entry 7 of 62 File: USPT Jun 25, 2002

US-PAT-NO: 6410271
DOCUMENT-IDENTIFIER: US 6410271 B1

TITLE: Generation of highly diverse library of expression vectors via homologous recombination in yeast

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 8. Document ID: US 6410246 B1

L8: Entry 8 of 62 File: USPT Jun 25, 2002

US-PAT-NO: 6410246
DOCUMENT-IDENTIFIER: US 6410246 B1

TITLE: Highly diverse library of yeast expression vectors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWMC
Draw Desc | Image

☐ 9. Document ID: US 6406863 B1

L8: Entry 9 of 62 File: USPT Jun 18, 2002

US-PAT-NO: 6406863
DOCUMENT-IDENTIFIER: US 6406863 B1

TITLE: High throughput generation and screening of fully human antibody repertoire in yeast

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWMC
Draw Desc | Image

☐ 10. Document ID: US 6395712 B1

L8: Entry 10 of 62 File: USPT May 28, 2002

US-PAT-NO: 6395712
DOCUMENT-IDENTIFIER: US 6395712 B1

TITLE: Sensitization of HER-2/neu overexpressing cancer cells to chemotherapy

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWMC
Draw Desc | Image

Generate Collection | Print

Term	Documents
DRUG.USPT.	90313
DRUGS.USPT.	72937
RESIST$	0
RESIST.USPT.	142043
RESISTA.USPT.	17
RESISTAACE.USPT.	1
RESISTAANCE.USPT.	4
RESISTAANT.USPT.	1
RESISTAAT.USPT.	1
RESISTABILITY.USPT.	69
RESISTABLATING.USPT.	1
(L7 AND DRUG AND RESIST$).USPT.	62

There are more results than shown above. Click here to view the entire set.

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Search Results - Record(s) 11 through 20 of 62 returned.

☐ 11. Document ID: US 6392019 B1

L8: Entry 11 of 62 File: USPT May 21, 2002

US-PAT-NO: 6392019
DOCUMENT-IDENTIFIER: US 6392019 B1

TITLE: Antibodies specific for EGF motif proteins

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 12. Document ID: US 6387657 B1

L8: Entry 12 of 62 File: USPT May 14, 2002

US-PAT-NO: 6387657
DOCUMENT-IDENTIFIER: US 6387657 B1

TITLE: WISP polypeptides and nucleic acids encoding same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 13. Document ID: US 6368596 B1

L8: Entry 13 of 62 File: USPT Apr 9, 2002

US-PAT-NO: 6368596
DOCUMENT-IDENTIFIER: US 6368596 B1

TITLE: Compositions and methods for homoconjugates of antibodies which induce growth arrest or apoptosis of tumor cells

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 14. Document ID: US 6339151 B1

L8: Entry 14 of 62 File: USPT Jan 15, 2002

US-PAT-NO: 6339151
DOCUMENT-IDENTIFIER: US 6339151 B1

TITLE: Enzyme catalyzed therapeutic agents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 15. Document ID: US 6329173 B1

L8: Entry 15 of 62 File: USPT Dec 11, 2001

US-PAT-NO: 6329173
DOCUMENT-IDENTIFIER: US 6329173 B1

TITLE: Method of intracellular binding target molecules

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 16. Document ID: US 6326356 B1

L8: Entry 16 of 62 File: USPT Dec 4, 2001

US-PAT-NO: 6326356
DOCUMENT-IDENTIFIER: US 6326356 B1

TITLE: Suppression of neu overexpression using a mini-E1A gene

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 17. Document ID: US 6322986 B1

L8: Entry 17 of 62 File: USPT Nov 27, 2001

US-PAT-NO: 6322986
DOCUMENT-IDENTIFIER: US 6322986 B1

TITLE: Method for colorectal cancer prognosis and treatment selection

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 18. Document ID: US 6306832 B1

L8: Entry 18 of 62 File: USPT Oct 23, 2001

US-PAT-NO: 6306832
DOCUMENT-IDENTIFIER: US 6306832 B1

TITLE: Peptide antiestrogen compositions and methods for treating breast cancer

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 19. Document ID: US 6261535 B1

L8: Entry 19 of 62 File: USPT Jul 17, 2001

US-PAT-NO: 6261535
DOCUMENT-IDENTIFIER: US 6261535 B1

TITLE: Diagnostic methods for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 20. Document ID: US 6245750 B1

L8: Entry 20 of 62 File: USPT Jun 12, 2001

US-PAT-NO: 6245750
DOCUMENT-IDENTIFIER: US 6245750 B1

TITLE: Enzyme catalyzed therapeutic agents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

Generate Collection | Print

Term	Documents
DRUG.USPT.	90313
DRUGS.USPT.	72937
RESIST$	0
RESIST.USPT.	142043
RESISTA.USPT.	17
RESISTAACE.USPT.	1
RESISTAANCE.USPT.	4
RESISTAANT.USPT.	1
RESISTAAT.USPT.	1
RESISTABILITY.USPT.	69
RESISTABLATING.USPT.	1
(L7 AND DRUG AND RESIST$).USPT.	62

There are more results than shown above. Click here to view the entire set.

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Search Results - Record(s) 21 through 30 of 62 returned.

☐ 21. Document ID: US 6235714 B1

L8: Entry 21 of 62 File: USPT May 22, 2001

US-PAT-NO: 6235714
DOCUMENT-IDENTIFIER: US 6235714 B1

TITLE: Methods for identifying inducers and inhibitors of proteolytic antibodies, compositions and their uses

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 22. Document ID: US 6210707 B1

L8: Entry 22 of 62 File: USPT Apr 3, 2001

US-PAT-NO: 6210707
DOCUMENT-IDENTIFIER: US 6210707 B1

TITLE: Methods of forming protein-linked lipidic microparticles, and compositions thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 23. Document ID: US 6197754 B1

L8: Entry 23 of 62 File: USPT Mar 6, 2001

US-PAT-NO: 6197754
DOCUMENT-IDENTIFIER: US 6197754 B1

TITLE: Suppression of tumor growth by a mini-E1A gene

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 24. Document ID: US 6180767 B1

L8: Entry 24 of 62 File: USPT Jan 30, 2001

US-PAT-NO: 6180767
DOCUMENT-IDENTIFIER: US 6180767 B1

TITLE: Peptide nucleic acid conjugates

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 25. Document ID: US 6156321 A

L8: Entry 25 of 62 File: USPT Dec 5, 2000

US-PAT-NO: 6156321
DOCUMENT-IDENTIFIER: US 6156321 A

TITLE: Tissue factor methods and compositions for coagulation and tumor treatment

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 26. Document ID: US 6146837 A

L8: Entry 26 of 62 File: USPT Nov 14, 2000

US-PAT-NO: 6146837
DOCUMENT-IDENTIFIER: US 6146837 A

TITLE: Cyanidin compositions and therapeutic and diagnostic uses therefor

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 27. Document ID: US 6132730 A

L8: Entry 27 of 62 File: USPT Oct 17, 2000

US-PAT-NO: 6132730
DOCUMENT-IDENTIFIER: US 6132730 A

TITLE: Combined tissue factor and factor VIIa methods and compositions for coagulation and tumor treatment

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 28. Document ID: US 6132729 A

L8: Entry 28 of 62 File: USPT Oct 17, 2000

US-PAT-NO: 6132729
DOCUMENT-IDENTIFIER: US 6132729 A

TITLE: Combined tissue factor and chemotherapeutic methods and compositions for coagulation and tumor treatment

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 29. Document ID: US 6072036 A

L8: Entry 29 of 62 File: USPT Jun 6, 2000

US-PAT-NO: 6072036
DOCUMENT-IDENTIFIER: US 6072036 A

TITLE: Method of intracellular binding of target molecules

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 30. Document ID: US 6051230 A

L8: Entry 30 of 62 File: USPT Apr 18, 2000

US-PAT-NO: 6051230
DOCUMENT-IDENTIFIER: US 6051230 A

TITLE: Compositions for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

Generate Collection | Print

Term	Documents
DRUG.USPT.	90313
DRUGS.USPT.	72937
RESIST$	0
RESIST.USPT.	142043
RESISTA.USPT.	17
RESISTAACE.USPT.	1
RESISTAANCE.USPT.	4
RESISTAANT.USPT.	1
RESISTAAT.USPT.	1
RESISTABILITY.USPT.	69
RESISTABLATING.USPT.	1
(L7 AND DRUG AND RESIST$).USPT.	62

There are more results than shown above. Click here to view the entire set.

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Search Results - Record(s) 31 through 40 of 62 returned.

☐ 31. Document ID: US 6045797 A

L8: Entry 31 of 62 File: USPT Apr 4, 2000

US-PAT-NO: 6045797
DOCUMENT-IDENTIFIER: US 6045797 A

TITLE: Treatment or diagnosis of diseases or conditions associated with a BLM domain

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 32. Document ID: US 6040290 A

L8: Entry 32 of 62 File: USPT Mar 21, 2000

US-PAT-NO: 6040290
DOCUMENT-IDENTIFIER: US 6040290 A

TITLE: Ligand growth factor gp30 that binds to the erbB-2 receptor protein and induces cellular responses

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 33. Document ID: US 6037134 A

L8: Entry 33 of 62 File: USPT Mar 14, 2000

US-PAT-NO: 6037134
DOCUMENT-IDENTIFIER: US 6037134 A

TITLE: Methods that detect compounds that disrupt receptor tyrosine kinase/GRB-7 complexes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 34. Document ID: US 6027892 A

L8: Entry 34 of 62 File: USPT Feb 22, 2000

US-PAT-NO: 6027892

DOCUMENT-IDENTIFIER: US 6027892 A

TITLE: Compositions and methods for reducing radiation and drug resistance in cells

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 35. Document ID: US 6004940 A

L8: Entry 35 of 62 File: USPT Dec 21, 1999

US-PAT-NO: 6004940
DOCUMENT-IDENTIFIER: US 6004940 A

TITLE: Intracellular targeting of endogenous proteins

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 36. Document ID: US 6004554 A

L8: Entry 36 of 62 File: USPT Dec 21, 1999

US-PAT-NO: 6004554
DOCUMENT-IDENTIFIER: US 6004554 A

TITLE: Methods for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 37. Document ID: US 6001583 A

L8: Entry 37 of 62 File: USPT Dec 14, 1999

US-PAT-NO: 6001583
DOCUMENT-IDENTIFIER: US 6001583 A

TITLE: Methods for disrupting GRB-7 complexes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 38. Document ID: US 5968748 A

L8: Entry 38 of 62 File: USPT Oct 19, 1999

US-PAT-NO: 5968748
DOCUMENT-IDENTIFIER: US 5968748 A

TITLE: Antisense oligonucleotide modulation of human HER-2 expression

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Draw Desc | Image

KWIC

☐ 39. Document ID: US 5965371 A

L8: Entry 39 of 62 File: USPT Oct 12, 1999

US-PAT-NO: 5965371
DOCUMENT-IDENTIFIER: US 5965371 A

TITLE: Method of intracellular binding of target molecules

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Draw Desc | Image

KWIC

☐ 40. Document ID: US 5965132 A

L8: Entry 40 of 62 File: USPT Oct 12, 1999

US-PAT-NO: 5965132
DOCUMENT-IDENTIFIER: US 5965132 A

TITLE: Methods and compositions for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Draw Desc | Image

KWIC

Generate Collection | Print

Term	Documents
DRUG.USPT.	90313
DRUGS.USPT.	72937
RESIST$	0
RESIST.USPT.	142043
RESISTA.USPT.	17
RESISTAACE.USPT.	1
RESISTAANCE.USPT.	4
RESISTAANT.USPT.	1
RESISTAAT.USPT.	1
RESISTABILITY.USPT.	69
RESISTABLATING.USPT.	1
(L7 AND DRUG AND RESIST$).USPT.	62

There are more results than shown above. Click here to view the entire set.

Display Format: TI Change Format

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Generate Collection | Print

Search Results - Record(s) 41 through 50 of 62 returned.

☐ 41. Document ID: US 5869618 A Feb 9, 1999

L8: Entry 41 of 62 File: USPT

US-PAT-NO: 5869618
DOCUMENT-IDENTIFIER: US 5869618 A

TITLE: Antibodies to ligand growth factors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 42. Document ID: US 5863538 A Jan 26, 1999

L8: Entry 42 of 62 File: USPT

US-PAT-NO: 5863538
DOCUMENT-IDENTIFIER: US 5863538 A

TITLE: Compositions for targeting the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
Draw Desc | Image

☐ 43. Document ID: US 5855866 A Jan 5, 1999

L8: Entry 43 of 62 File: USPT

US-PAT-NO: 5855866
DOCUMENT-IDENTIFIER: US 5855866 A

TITLE: Methods for treating the vasculature of solid tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 44. Document ID: US 5851829 A Dec 22, 1998

L8: Entry 44 of 62 File: USPT

US-PAT-NO: 5851829
DOCUMENT-IDENTIFIER: US 5851829 A

TITLE: Method of intracellular binding of target molecules

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 45. Document ID: US 5840525 A

L8: Entry 45 of 62 File: USPT Nov 24, 1998

US-PAT-NO: 5840525
DOCUMENT-IDENTIFIER: US 5840525 A

TITLE: Nucleic acids, vectors and host cells encoding heregulin

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 46. Document ID: US 5837523 A

L8: Entry 46 of 62 File: USPT Nov 17, 1998

US-PAT-NO: 5837523
DOCUMENT-IDENTIFIER: US 5837523 A

TITLE: Compositions and methods of treating tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 47. Document ID: US 5830660 A

L8: Entry 47 of 62 File: USPT Nov 3, 1998

US-PAT-NO: 5830660
DOCUMENT-IDENTIFIER: US 5830660 A

TITLE: Tumorigenesis protein

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 48. Document ID: US 5814315 A

L8: Entry 48 of 62 File: USPT Sep 29, 1998

US-PAT-NO: 5814315
DOCUMENT-IDENTIFIER: US 5814315 A

TITLE: Methods for the suppression of neu mediated phenotype in tumors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 49. Document ID: US 5808036 A

L8: Entry 49 of 62 File: USPT Sep 15, 1998

US-PAT-NO: 5808036
DOCUMENT-IDENTIFIER: US 5808036 A

TITLE: Stem-loop oligonucleotides containing parallel and antiparallel binding domains

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWMC
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☐ 50. Document ID: US 5792608 A

L8: Entry 50 of 62 File: USPT Aug 11, 1998

US-PAT-NO: 5792608
DOCUMENT-IDENTIFIER: US 5792608 A

TITLE: Nuclease stable and binding competent oligomers and methods for their use

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWMC
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Term	Documents
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DRUGS.USPT.	72937
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RESISTAANCE.USPT.	4
RESISTAANT.USPT.	1
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☐ 61. Document ID: US 5514546 A

L8: Entry 61 of 62 File: USPT May 7, 1996

US-PAT-NO: 5514546
DOCUMENT-IDENTIFIER: US 5514546 A

TITLE: Stem-loop oligonucleotides containing parallel and antiparallel binding domains

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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☐ 62. Document ID: US 5367060 A

L8: Entry 62 of 62 File: USPT Nov 22, 1994

US-PAT-NO: 5367060
DOCUMENT-IDENTIFIER: US 5367060 A

TITLE: Structure, production and use of heregulin

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC
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(L7 AND DRUG AND RESIST$).USPT.	62

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L8: Entry 62 of 62 File: USPT Nov 22, 1994

DOCUMENT-IDENTIFIER: US 5367060 A
TITLE: Structure, production and use of heregulin

Detailed Description Text (44):
HRG also includes NTD-GFD having its C-terminus at one of the first about 1 to 3 extracellular domain residues (QKR, residues 240-243, HRE-.alpha., FIG. 15) or first about 1-2 transmembrane region residues. In addition, in some HRG-GFD variants the codons are modified at the GFD-transmember proproteolysis site by substitution, insertion or deletion. The GFD proteolysis site is the domain that contains the GFD C-terminal residue and about 5 residues N- and 5 residues C-terminal from this residue. At this time neither the natural C-terminal residue for HRG-.alpha. or HRG-.beta. has been identified, although it is known that Met-227 terminal and Val-229 terminal HRG-.alpha.-GFD are biologically active. The native C-terminus for HRG-.alpha.-GFD is probably Met-227, Lys-228, Val-229, Gln-230, Asn-231 or GIn-232, and for HRG .beta..sub.1 -.beta..sub.2 -GFD is probably Met-226, Ala-227, Ser-228, Phe-229, Trp-230, Lys 231or (for HRG-.beta..sub.1) K240 or (for HRG-.beta..sub.2) K2246. The native C-terminus is determined readily by C-terminal sequencing, although it is not critical that HRG-GFD have the native terminus so long as the GFD sequence possesses the desired activity. In some embodiments of HRG-GFD variants, the amino acid change(s) in the CTP are screened for their ability to resist proteolysis in vitro and inhibit the protease responsible for generation of HRG-GFD.

Detailed Description Text (109):
Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Detailed Description Text (110):
One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1: 327,1982), mycophenolic acid (Mulligan et al., Science 209: 1422,1980) or hygromycin (Sugden et al., Mol. Cell. Biol. 5: 410-413,1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Detailed Description Text (112):
For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl.

Acad. Sci. USA, 77: 4216, 1980. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding HRG. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,0603. Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding HRG, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418 (see U.S. Pat. No. 4,965,199).

Detailed Description Text (127):
For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res. 9: 309 (1981) or by the method of Maxam et al., Methods in Enzymology 65: 499 (1980).

Detailed Description Text (140):
The mammalian host cells used to produce HRG of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58:44 (1979), Barnes and Sato, Anal. Biochem., 102:255 (1980), U.S Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430, WO 87/00195, U S Pat. Re. 30,985; or copending U.S. Ser. No. 07/592,107 or 07/592,141, both filed on 3 October 1990, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin.TM. drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Detailed Description Text (162):
HRG optionally is fused with a polypeptide heterologous to HRG. The heterologous polypeptide optionally is an anchor sequence such as that found in the decay accelerating system (DAF); a toxin such as ricin, pseudomonas exotoxin, gelonin, or other polypeptide that will result in target cell death. These heterologous polypeptides are covalently coupled to HRG through side chains or through the terminal residues. Similarly, HRG is conjugated to other molecules toxic or inhibitory to a target mammalian cell, e.g. such as tricothecenes, or antisense DNA that blocks expression of target genes.

Detailed Description Text (171):
HRG also is entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate]microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences. 16th edition, Osol, A., Ed., (1980).

Detailed Description Text (198):
Antibodies that are capable of binding to proteolytic processing sites are of

particular interest. They are produced either by immunizing with an HRG fragment that includes the CTP processing site, with intact HRG, or with HRG-NTD-GFD and then screening for the ability to block or inhibit proteolytic processing of HRG into the NTD-GFD fragment by recombinant host cells or isolated cell lines that are otherwise capable of processing HRG to the fragment. These antibodies are useful for suppressing the release of NTD-GFD and therefore are promising for use in preventing the release of NTD-GFD and stimulation of the HER-2 receptor. They also are useful in controlling cell growth and replication. Anti-GFD antibodies are useful for the same reasons, but may not be as efficient biologically as antibodies directed against a processing site.

Detailed Description Text (199):
Antibodies are selected that are capable of binding only to one of the members of the HRG family, e.g. HRG-alpha or any one of the HRG-beta isoforms. Since each of the HRG family members has a distinct GFD-transmembrane domain cleavage site, antibodies directed specifically against these unique sequences will enable the highly specific inhibition of each of the GFDs or processing sites, and thereby refine the desired biological response. For example, breast carcinoma cells which are HER-2 dependent may in fact be activated only by a single GFD isotype or, if not, the activating GFD may originate only from a particular processing sequence, either on the HER-2 bearing cell itself or on a GFD-generating cell. The identification of the target activating GFD or processing site is a straight-forward matter of analyzing HER-2 dependent carcinomas, e.g., by analyzing the tissues for the presence of a particular GFD family member associated with the receptor, or by analyzing the tissues for expression of an HRG family member (which then would serve as the therapeutic target). These selective antibodies are produced in the same fashion as described above, either by immunization with the target sequence or domain, or by selecting from a bank of antibodies having broader specificity.

Detailed Description Text (200):
As described above, the antibodies should have high specificity and affinity for the target sequence. For example, the antibodies directed against GFD sequences should have greater affinity for the GFD than GFD has for the HER-2 receptor. Such antibodies are selected by routine screening methods.

Detailed Description Text (277):
Heregulin-.beta.2 and -.beta.3 variants were isolated in order to obtain cDNA clones that extend further in the 5' direction. A specifically primed cDNA library was constructed in .lambda.gt10 by employing the chemically synthesized antisense primer 3'CCTTCCCGTTCTTCTTCCTCGCTCC (SEQ ID No.21). This primer is located between nucleotides 167-190 in the sequence of .lambda.her16 (FIG. 4). The isolation of clone .lambda.5'her13 (not to be confused with .lambda.her13) was achieved by hybridizing a synthetic DNA probe corresponding to the 5' end of .lambda.her16 under high stringency conditions with the specifically primed cDNA library. The nucleotide sequence of .lambda.5'her13 is shown in FIG. 11 (SEQ ID NO:22). The 496 base pair nucleotide sequence of .lambda.5'her13 is homologous to the sequence of .lambda.her16 between nucleotides 309-496 of .lambda.5'her13 and 3-190 of .lambda.her16. .lambda. 5'her13 extends by 102 amino acids the open reading frame of .lambda.her16.

L8: Entry 61 of 62 File: USPT May 7, 1996

DOCUMENT-IDENTIFIER: US 5514546 A
TITLE: Stem-loop oligonucleotides containing parallel and antiparallel binding domains

Brief Summary Text (2):
The present invention provides stem-loop oligonucleotides capable of strong binding to a target DNA or RNA. Moreover, stem-loop oligonucleotides are resistant to nucleases and bind to a target with high selectivity and affinity. Such strong binding allows the present stem-loop oligonucleotides to be utilized in a variety of ways. For example, stem-loop oligonucleotides can be labeled for use as probes to detect or isolate a target nucleic acid. Stem-loop oligonucleotides can also be transcribed within, or administered to, a cell to provide in vivo regulators of DNA replication, RNA transcription, protein translation, reverse transcription, and other processes involving nucleic acid templates.

Brief Summary Text (7):
Furthermore, there has been great interest recently in developing oligonucleotides as regulators of cellular nucleic acid biological function. This interest arises from observations on naturally occurring complementary, or antisense, RNA used by some cells to control protein expression. However, the development of oligonucleotides for in vivo regulation of biological processes has been hampered by several long-standing problems, including the low binding affinity and nuclease sensitivity of linear oligonucleotides.

Brief Summary Text (9):
Splicing of a pre-mRNA transcript essential for Herpes Simplex virus replication has also been inhibited with a linear oligonucleotide which was complementary to an acceptor splice junction. In this instance, a methylphosphonate linkage was employed in the linear oligonucleotide to increase its nuclease resistance. Addition of this chemically-modified oligonucleotide to the growth medium caused reduction in protein synthesis and growth of uninfected cells, most likely because of toxicity problems occurring at high oligonucleotide concentrations (Smith et al., 1986, Proc. Natl. Acad. Sci. USA 83:2787-2791).

Brief Summary Text (16):
The present invention provides stem-loop oligonucleotides which have many of the desirable attributes of circular oligonucleotides, e.g. nuclease resistance (Tang et al., 1993, Nucleic Acids Res. 21:2729-2735). However the present stem-loop oligonucleotides are much simpler to make both in vivo and in vitro. Moreover, the present stem-loop oligonucleotides bind target via both Watson-Crick and non-Watson-Crick hydrogen bonding. The present stem-loop oligonucleotides bind with strong affinity and high selectivity to their targeted nucleic acids.

Brief Summary Text (27):
In another embodiment the present invention provides a method of specific cell type drug delivery which includes administering an oligonucleotide of the present invention to an animal wherein the oligonucleotide has a covalently linked drug.

Detailed Description Text (2):
The present invention relates to stem-loop oligonucleotides which can bind to nucleic acid targets with high affinity and selectivity. Such strong, selective binding of these oligonucleotides to either single- or double-stranded DNA or RNA

targets provides a variety of uses, including methods of regulating such biological processes as DNA replication, RNA transcription, RNA splicing and processing and protein translation. Similarly, the strong binding properties of these stem-loop oligonucleotides makes these oligonucleotides ideal diagnostic probes or markers to localize, for example, specific sites in a chromosome or other DNA or RNA molecules. Additionally, the present stem-loop oligonucleotides are useful for isolation of complementary nucleic acids or for sequence-specific delivery of drugs or other molecules into cells.

Detailed Description Text (72):
The expression vectors of the present invention can also encode selectable markers. Selectable markers are genetic functions that confer an identifiable trait upon a host cell so that cells transformed with a vector carrying the selectable marker can be distinguished from non-transformed cells. Inclusion of a selectable marker into a vector can also be used to ensure that genetic functions linked to the marker are retained in the host cell population. Such selectable markers can confer any easily identified dominant trait, e.g. drug resistance, the ability to synthesize or metabolize cellular nutrients and the like.

Detailed Description Text (78):
Moreover, according to the present invention, the loop domains which do not encode a P or AP domain do not have to be composed of nucleotide bases. Non-nucleotide loop domains can make the present stem-loop oligonucleotides less expensive to produce. More significantly, stem-loop oligonucleotides with non-nucleotide loop domains are more resistant to nucleases and therefore have a longer biological half-life than linear oligonucleotides. Furthermore, non-nucleotide loop domains having no charge, or a positive charge, can be used to promote binding by eliminating negative charge repulsions between the loop and target. In addition, stem-loop oligonucleotides having uncharged or hydrophobic non-nucleotide loop domains can penetrate cellular membranes better than stem-loop oligonucleotides with nucleotide loops.

Detailed Description Text (82):
The present invention further contemplates derivatization of the subject oligonucleotides with agents that can cleave or modify the target nucleic acid or other nucleic acid strands associated with or in the vicinity of the target. For example, viral DNA or RNA can be targeted for destruction without harming cellular nucleic acids by administering a stem-loop oligonucleotide complementary to the targeted nucleic acid which is linked to an agent that, upon binding, can cut or render the viral DNA or RNA inactive. Nucleic acid destroying agents that are contemplated by the present invention as having cleavage or modifying activities include, for example, RNA and DNA nucleases, ribozymes that can cleave RNA, azidoproflavine, acridine, EDTA/Fe, chloroethylamine, azidophenacyl, psoralen and phenanthroline/Cu. Uhlmann et al. (1990, Chemical Reviews 90:543-584) and Beaucage et al. (1993, Tetrahedron 49:1925-1963) provide further information on the use of such agents and methods of derivatizing oligonucleotides that can be adapted for use with the subject stem-loop oligonucleotides.

Detailed Description Text (83):
Therefore, derivatization of the subject stem-loop oligonucleotides with reporter molecules, nucleic acid destroying agents, drugs, groups that facilitate cellular uptake or groups that facilitate target binding can be done by any of the procedures known to one skilled in the art. Moreover, the desired groups can be added to nucleotides before or after synthesis of the oligonucleotide. For example, these groups can be linked to the 5-position of T or C and these modified T and C nucleotides can be used for synthesis of the present stem-loop oligonucleotides.

Detailed Description Text (85):
The present invention further contemplates modification in the phosphodiester backbone of stem-loop oligonucleotides, e.g. as described in Dolnick (1991, Cancer Invest. 9:185-194). Such modifications can aid uptake of the oligonucleotide by cells or can extend the biological half-life of such nucleotides. For example, stem-loop oligonucleotides may penetrate the cell membrane more readily if the negative charge on the internucleotide phosphate is eliminated. This can be done by replacing the negatively charged phosphate oxygen with a methyl group, an amine or by changing the phosphodiester linkage into a phosphotriester linkage by addition of

an alkyl group to the negatively charged phosphate oxygen. Alternatively, one or more of the phosphate atoms which is part of the normal phosphodiester linkage can be replaced. For example, NH--P, CH.sub.2 --P or S--P linkages can be formed. Accordingly, the present invention contemplates using methylphosphonates, phosphorothioates, phosphorodithioates, phosphotriesters and phosphorusboron (Sood et al., 1990, J. Am. Chem. Soc. 112:9000) linkages. The phosphodiester group can be replaced with siloxane, carbonate, acetamidate or thioether groups. These modifications can also increase the resistance of the subject oligonucleotides to nucleases. Methods for synthesis of oligonucleotides with modified phosphodiester linkages are reviewed by Uhlmann et al.

Detailed Description Text (88):
The present invention contemplates a variety of utilities for the subject stem-loop oligonucleotides which are made possible by their selective and stable binding properties with both single- and double-stranded targets. Some utilities include, but are not limited to: regulating biosynthesis of a DNA, RNA or protein encoded by providing a stem-loop oligonucleotide to a template for the DNA, the RNA or the protein; use of stem-loop oligonucleotides of defined sequence, bound to a solid support, for affinity isolation of complementary nucleic acids; use of the subject oligonucleotides to provide sequence specific stop signals during polymerase chain reaction (PCR); covalent attachment of a drug, drug analog or other therapeutic agent to stem-loop oligonucleotides to allow cell type specific drug delivery; and labeling stem-loop oligonucleotides with a detectable reporter molecule for localizing, quantitating or identifying complementary target nucleic acids.

Detailed Description Text (111):
Moreover, the present methods of regulating the biosynthesis of a DNA, RNA or protein can also be used to inhibit cellular oncogenes. Cellular protooncogenes are thought to have a normal role in cellular replication which is improperly executed when the protooncogene becomes mutated. Rather than controlling cellular growth, mutated protooncogenes, or oncogenes, can contribute to uncontrolled cellular growth and thereby increase the likelihood of developing cancer. The present methods can inhibit the transcription and translation of oncogenes such as, for example, oncogenes like the c-abl, bcr-abl, bcl-2, c-cbl, c-dbl, c-erb, c-ets, c-fgf, c-fms, c-fos, c-has/bas, her-2 neu, c-int, c-jun, c-kit, c-mas, c-met, c-mos, c-myb, c-myc, N-myc, p53, ras, c-Ha-ras, c-rel, c-ret, c-ros, c-sec, c-sis, c-ski, c-snoA, c-snoN, c-spi, c-src, c-syn, c-trk, c-vav and c-yes.

Detailed Description Text (115):
The present invention also contemplates using the subject stem-loop oligonucleotides for targeting drugs to specific cell types. Such targeting can allow selective destruction or growth of particular cell types, e.g. inhibition of tumor cell growth can be attained. To target a drug to a specific cell type the skilled artisan takes advantage of the fact that different cell types express different genes, so that the concentration of a particular mRNA can be greater in one cell type relative to another cell type. An mRNA which is present in higher concentrations in the cell to which the drug is to be delivered is a suitable target mRNA. Cells with high concentrations of target mRNA are targeted for drug delivery by administering to the cell a stem-loop oligonucleotide which is complementary to the target mRNA and which has a covalently linked drug.

Detailed Description Text (118):
Stem-loop oligonucleotide:solid supports can be used, for example, to isolate poly(A).sup.+ mRNA from total cellular or viral RNA when the stem-loop oligonucleotide has P and AP domain poly(dT) or poly(U) sequences. Stem-loop oligonucleotides are ideally suited to applications of this type because they are nuclease resistant and bind target nucleic acids so strongly.

Detailed Description Text (211):
Nuclease Resistance

Detailed Description Text (212):
The nuclease resistance of stem-loop and linear oligonucleotides are compared when these oligonucleotides are incubated in human plasma for varying time periods. Stem-loop and linear oligonucleotides having similar numbers of nucleotides are

incubated at about a 50 .mu.M concentration in plasma at 37.degree. C. Aliquots are removed at various time points and cleavage products are separated by gel electrophoresis. Nuclease resistance is assessed by observing whether degradation products are evident on the gels.

L8: Entry 60 of 62 File: USPT May 21, 1996

DOCUMENT-IDENTIFIER: US 5518885 A
TITLE: ERBB2 promoter binding protein in neoplastic disease

Brief Summary Text (12):
A recent study.sup.43 of 209 consecutive female patients with invasive operable breast cancer from a defined urban population observed for a median of 30 years demonstrated that fifty-five patients (26%) had cancer and a positive ERBB2 oncoprotein stain reaction. They had significantly reduced 10 and 25 years survival rates as compared with those patients who had a negative stain reaction in their cancer (31% versus 48% and 31% versus 39% respectively with a P value=0.004). ERBB2 gene expression was also found to be associated with reduced survival among patients who had axillary nodal metastases (P value=0.003) but not among those patients who did not have metastases. ERBB2 expression was related to the ductal histologic type, poor histologic grade and high mitotic count, but not to tumor size, axillary nodal status, DNA ploidy or S-phase fraction. In a multivariate analysis among patients with nodal metastases, ERBB2 expression was found to be an independent prognostic factor (P value=0.004) that predicted poor survival. Based on these data, it was concluded that ERBB2 oncoprotein expression has long-term prognostic significance for predicting poor survival in breast cancer and it has an independent prognostic value among patients who presented with axillary nodal metastases. The mean survival time for the women with ERBB2 expressing group is only 29 months compared to the mean survival time of 110 months of the women with nonexpressing cancer. The difference between the survival curve is the greatest at approximately five years from the diagnosis (37% versus 64%) and diminished toward the end of the follow-up, which indicates that ERBB2 expressing cancers usually progress rapidly and are fatal. The result that ERBB2 expression predicts poor survival is contradictory to the opinion that it could only be a marker for drug resistance,.sup.44 not a marker for poor prognosis.

Brief Summary Text (42):
Finally, the present invention provides a method of inhibiting a biological activity mediated by HPBF comprising preventing the HPBF from binding to the promoter region of the ERBB2 gene sequence wherein the binding to the promoter region is prevented by an antisense nucleotide sequence or wherein the binding to the promoter region is prevented by a nongenomic nucleic acid sequence to which the HPBF binds.

Detailed Description Text (14):
The binding of antibodies to a solid support substrate is also well known in the art. (See, for example, Harlow and Lane, Antibodies; A Laboratory Manual, Cold Spring, Harbor Laboratory, Cold Spring Harbor, N.Y., 1988). The detectable moieties contemplated with the present invention can include fluorescent, enzymatic and radioactive markers. Therapeutic drugs contemplated with the present invention can include cytotoxic moieties such as ricin A chain, diphtheria toxin and chemotherapeutic compounds. Such therapeutic drugs can be utilized for killing cancer cells expressing HPBF.

Detailed Description Text (36):
In one example, the present invention provides a method of inhibiting a biological activity mediated by HPBF comprising preventing the HPBF from binding to the promoter region of the ERBB2 gene sequence wherein the binding to the promoter region is prevented by an antisense nucleotide sequence. The antisense oligonucleotide can be generated using well known nucleic acid synthesis methods as

demonstrated in the Examples.

Detailed Description Text (38):
A method to inhibit a biological activity of HPBF and decrease ERBB2 activity can use antisense or triplex oligonucleotide analogues or expression constructs. This entails introducing into the cell a nucleic acid sufficiently complementary in sequence so as to selectively hybridize to the target gene or message. Triplex inhibition relies on the transcriptional inhibition of the target gene and can be extremely efficient since only a few copies per cell are required to achieve complete inhibition. Antisense methodology on the other hand inhibits the normal processing, translation or half-life of the target message. Such methods are well known to one skilled in the art.

Detailed Description Text (39):
Although longer sequences can be used to achieve inhibition, antisense and triplex methods generally involve the treatment of cells or tissues with a relatively short oligonucleotide. The oligonucleotide can be either deoxyribo- or ribonucleic acid and must be of sufficient length to form a stable duplex or triplex with the target RNA or DNA at physiological temperatures and salt concentrations. It should also be of sufficient complementarity to selectively hybridize to the target nucleic acid. Oligonucleotide lengths sufficient to achieve this specificity are generally about 12 to 60 nucleotides long, preferably about 18 to 32 nucleotides long. In addition to length, hybridization specificity is also influenced by GC content and primary sequence of the oligonucleotide. Such principles are well known in the art and can be routinely determined by one who is skilled in the art.

Detailed Description Text (40):
The composition of the antisense or triplex oligonucleotides can also influence the efficiency of inhibition. For example, it is preferable to use oligonucleotides that are resistant to degradation by the action of endogenous nucleases. Nutlease resistance will confer a longer in vivo half-life onto the oligonucleotide and therefore increase its efficacy by reducing the required dose. Greater efficacy can also be obtained by modifying the oligonucleotide so that it is more permeable to cell membranes. Such modifications are well known in the art and include the alteration of the negatively charged phosphate backbone of the oligonucleotide to uncharged atoms such as sulfur and carbon. Specific examples of such modifications include oligonucleotides that contain methylphosphonate and thiophosphonate moieties in place of phosphate. These modified oligonucleotides can be applied directly to the cells or tissues to achieve entry into the cells and inhibition of HPBF activity. Other types of modifications exist as well and are known to one skilled in the art.

Detailed Description Text (41):
Recombinant methods known in the art can also be used to achieve the antisense or triplex inhibition of a target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity. Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the antisense or triplex sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

Detailed Description Text (42):
The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, N.Y. (1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), and include, for example, stable or transient transfection,

lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the antisense vectors to specific cell types in vivo or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

Detailed Description Text (43):
A specific example of a DNA viral vector for introducing and expressing antisense nucleic acids is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences such as antisense sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an in vitro or ex vivo culture of cells, a tissue or a human subject.

Detailed Description Text (44):
Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or antisense sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

Detailed Description Text (48):
HPBF antisense-encoding viral vectors can be administered in several ways to obtain expression and therefore decrease the activity of HPBF in cells affected by the disease or pathological condition. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into the spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

Detailed Description Text (49):
An alternate mode of administration of HPBF antisense-encoding vectors can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the tumor with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve HPBF expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Detailed Description Text (50):
In addition to the antisense methods described above, other methods can be used as

well to decrease the activity of HPBF and achieve the down regulation of ERBB2 activity. For example, oligonucleotides which compete for the HPBF binding site within the ERBb2 regulatory elements can be used to competitively inhibit HPBF binding to ERBB2. Such oligonucleotides can be, for example, methylphosphonates and thiophosphonates which permeate the cell membrane. Alternatively, vectors which express such sequences or contain the HPBF binding element can also be used to achieve the same result as the oligonucleotides. Modes of administration for the competitive inhibition are similar to that described above for the antisense vectors and oligonudeotides.

Detailed Description Text (52):
Stably transformed cell lines expressing HPBF can be constructed in several ways. One example of such a technique is integrating genetic material known to encode HPBF into the chromosome of a host cell. Such integration, usually mediated through transfection of the DNA by DEAE Dextran, Calcium Phosphate precipitation, or via liposome encapsulation, can be coupled to the introduction of genes utilized to enhance gene expression. For example, the gene for the metabolic inhibitor, dihydrofolate reductase can be selected as the cotransfecting DNA to achieve DNA amplification and therefore enhanced or activated gene expression. In such a system, co-transfected cells are treated with methotrexate, a known inhibitor of dihydrofolate reductase. Cells resistant to methotrexate obtain this resistance by amplifying the numbers of dihydrofolate reductase genes. Genes other than the dihydrofolate gene are amplified as well .sup.104.

Detailed Description Text (169):
43. Toikkanen S., Helin H., Isola J., Joensuu H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. J. Clin. Oncol. 10:1044-1048, 1992.

L8: Entry 59 of 62 File: USPT Jun 24, 1997

DOCUMENT-IDENTIFIER: US 5641484 A
TITLE: Methods for the suppression of neu mediated tumors by adenoviral E1A and SV40 large T antigen

Brief Summary Text (5):
The c-erbB gene encodes the epidermal growth factor receptor (EGFr) and is highly homologous to the transforming gene of the avian erythroblastosis virus (Downward et al., 1984). The c-erbB gene is a member of the tyrosine-specific protein kinase family to which many proto-oncogenes belong. The c-erbB gene has recently been found to be similar, but distinct from, an oncogene referred to variously as c-erbB-2, HER-2 or neu oncogene (referred to herein simply as the neu oncogene), now known to be intimately involved in the pathogenesis of cancers of the human female breast and genital tract.

Brief Summary Text (58):
The term "E1A gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an E1A gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "E1A gene" may also comprise any combination of associated control sequences.

Brief Summary Text (62):
The term "LT gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an LT gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "LT gene" may also comprise any combination of associated control sequences.

Drawing Description Text (26):
FIG. 10A. E1A gene products inhibited the cell motility of the neu-transformed 3T3 cells. N-E1A: NIH3T3 cells transfected with E1A; B-neo: B104-1-1 cells transfected with neomycin resistant gene; B-E1A-1 to 5: five independent cell lines generated by transfecting E1A gene into B104-1-1 cells. The motility assays were carried out by using a transwell unit with 5 .mu.m pore size polycarbonate filter in 24 well cluster plate (Costar). Lower compartment of the transwell contained 600 .mu.l of one of the chemoattractants: 20 .mu.m fibronectin (FN) or 100 .mu.m FN dissolved in DMEM/F12, or hepatic endothelial cell conditioned media (HSE), or DMEM/F12 medial only as negative control. The cells (3.times.10.sup.4 /0.1 ml in DMEM/F12) were plated in the upper compartment and incubated for 6 hrs at 37.degree. C. in a humidified 5% CO.sub.2 atmosphere. After the incubation, the filters were fixed with 3% glutaraldehyde in PBS buffer and stained with Geimsa. Each sample was assayed in triplicate and cell motility was measured by counting the number of cells that had migrated to the lower side of the filter. At least four HPFs were counted per filter. The number of cells migrated to DMEM/F12 has been deducted from each sample to eliminate the background and all the assays were done in triplicates.

Drawing Description Text (56):
FIG. 20C. Effect of K1 on the transforming activity of activated neu. One mg of cNeu-104 was cotransfected with 2 mg of K1 and 0.1 mg of pSV2neo into Rat-1 cells. pSV2E was used as filler plasmid so that a final 5 mg DNA was transfected into cells. Cells were split 1:4 48 hours after transfection and duplicate plates were subsequently grown in regular medium (DMEM/F12 plus 10% calf serum) or regular medium supplemented with 250 mg/mL G418. Foci and G418-resistant colonies were stained and counted after 3-4 weeks. Results are expressed as ratio of foci to that

of G418-resistant colonies from each transfection to correct for transfection efficiency. The number of foci from transfecting cNeu-104 alone was set at 100%.

Detailed Description Text (3):
The activated neu oncogene contains a single amino acid substitution in the transmembrane domain and possesses an increased tyrosine kinase activity when compared to its normal counterpart. Furthermore, it has demonstrated that amplification of the neu protooncogene facilitates oncogenic activation by a single point mutation (Hung et al., 1989). the human homologue of the rat neu oncogene, also named as HER-2 or c-erbB2, has been shown to be amplified/overexpressed in 25-30% of human primary breast cancers and ovarian cancers (Hung et al., 1988; Slamon et al., 1987). Breast cancer patients with neu overexpression show a significantly lower overall survival rate and a shorter time to relapse than those patients without neu overexpression, suggesting that neu overexpression may be used as a prognostic factor (Id.). Amplification/overexpression of the human neu gene has also been shown to correlate with the number of axillary lymph nodes positive for metastasis in breast cancer patients (Id.). These studies strongly suggest that the neu oncogene may play an important role in malignant transformation and metastasis.

Detailed Description Text (51):
The B104-1-1 cell line, an NIH3T3 transfectant that has approximately 10-20 copies of mutation-activated genomic neu oncogene has been shown to be highly transforming and tumorigenic (Bargmann et al., 1986; Stern et al., 1986). For the present studies, B104-1-1 cells and control NIH3T3 cells were transfected with either E1A plasmids expressing adenovirus-5 E1A gene, (pE1A), or a derivative plasmid containing only the E1A promoter without the E1A coding sequence (pE1Apr). Cells were cotransfected with pSV2neo plasmids carrying a neomycin resistant marker gene (Southern et al., 1982).

Detailed Description Text (52):
The transfections were carried out with the modified calcium phosphate precipitation procedure of Chen and Okayama (1988). In each transfection, 5.times.10.sup.5 B104-1-1 cells or NIH3T3 cells (2.times.10 cm dishes) were seeded 24 h before transfection. The cells were transfected with either 10 .mu.g of the E1A expressing pE1A plasmid DNA or its derivative pE1Apr plasmid DNA, along with 1 .mu.g of pSV2-neo plasmid DNA (Southern et al., 1982). Approximately 14 h post-transfection, cells were washed and cultured in fresh medium for 24 h and split at a 1:10 ratio. The cells were then grown in selection medium containing 500 .mu.g/ml of G418 for 2-3 weeks and individual G418 resistant colonies were cloned using cloning rings and expanded to mass culture.

Detailed Description Text (75):
An increase in cell motility has been shown to correlate with a higher metastatic potential. Therefore, a motility assay, which measures the migration of the tested cell to a chemo-attractant, fibronectin or hepatic sinusoidal endothelial cell conditioned media, was performed. As shown in FIG. 10A, all of the B-E1A transfectants showed decreased migration rate to different chemoattractants than that of B-neo cell line, which are B104-1-1 cells transfected with neomycin-resistant (neo.sup.r) gene alone. The N-E1A cells also had a low migration rate which is comparable to that of N1H3T3 cells.

Detailed Description Text (82):
The E1A-expressing plasmid was cotransfected into SKOV3.ip1 cells together with the pSV2-neo plasmid carrying the neomycin-resistance marker gene, thus generating the E1A-expressing ovarian carcinoma stable transfectants. The G418-resistant clones were selected and expanded into cell lines, which were designated ip1.E1A cell lines. The same approach was used to select control cell lines, in which the pE1Ad1343 plasmids containing a 2-base pair frameshift deletion in the E1A coding sequence and producing nonfunctional protein products were introduced into the SKOV3.ip1 cells to generate the ip1.Efs cell line.

Detailed Description Text (83):
It was possible that some of the stable transfectants selected by this cotransfection strategy only harbored the neomycin resistance gene but not the E1A gene. Therefore, to identify those ip1.E1A transfectants that integrated the E1A

gene and actually produced E1A proteins, immunoblot analysis with anti-E1A antibodies was performed (FIG. 12A). Two of the ip1.E1A transfectants expressed multiple species of E1A proteins as described by Harlow et al., (1985), whereas the control ip1.Efs cell line, as expected, did not express E1A proteins.

Detailed Description Text (109):
NIH 3T3, B104-1-1 and Rat-1 cells were maintained in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 10% calf serum and 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells transfected with the drug selection plasmid, pSV2neo, were grown in the above media containing 400 mg/mL G418.

Detailed Description Text (113):
The drug selection plasmid pSV2neo was cotransfected with plasmids encoding LT into B104-1-1 cells. The transfected plates were trypsinized after 48 h and split into 4 plates and subsequently maintained in media containing 400 mg/mL G418. After 3 weeks, colonies were isolated and established in media containing G418).

Detailed Description Text (121):
Focus forming assay was carried out as described (Yu et al., 1992). The cosmid clone, cNeu-104 (Hung et al., 1986), contains 30 kb of activating genomic rat neu including 2.2 kb of the neu promoter. cNeu-104 (0.5 mg) was cotransfected into normal fibroblasts (Rat-1 cells) with 0.1 mg of the drug selection plasmid, pSV2neo, and 5-10 mg plasmids encoding mutant LT (pK1) or control filler plasmid, pSV2E. Cells were trypsinized and split into 4 plates 48 h after transfection. Two plates were maintained in regular media while the other 2 plates were maintained in media supplemented with G418. For cells kept in regular media for 3 weeks, foci of transformed cells appeared on a background monolayer of nontransformed cells. G418 resistant colonies appeared for plates maintained in G418 media. Foci and G418 resistant colonies were stained with 1% crystal violet and counted. To normalize for transfection efficiency, the number of foci formed for each transfection was divided by the number of G418 colonies obtained.

Detailed Description Text (124):
To test the effect of LT in cells that overexpress neu encoded p185, plasmids encoding LT, pZ189 (driven by the SV 40 promoter), together with pSV2neo (plasmids encoding the gene for neomycin resistance) were cotransfected into B104-1-1 cells. B104-1-1 cells are derived from NIH 3T3 cells transformed by the mutation-activated genomic rat neu oncogene (Shih et al., 1981; Hung et al., 1986). B104-1-1 cells express high levels of activated neu encoded p185, are phenotypically transformed (Padhy et al., 1982; Shih et al., 1981), highly tumorigenic (Yu et al., 1991; Hung et al. 1989) and have increased metastatic potential (Yu et al., 1991; Yu et al. 1992). The LT-transfected and G418 resistant B104-1-1 cells were cloned after 3 weeks and 2 cell lines expanded from the clones (named BTn14 and BTn16 cell lines) were analyzed for expression of LT and p185. Immunoblotting of cell lysates for LT using anti-LT antibody (SV 40 T-Ag, Ab-2, Oncogene Science), showed 2 bands of molecular weights less than 111 kd indicating expression of LT in BTn14 and BTn16 cell lines (FIG. 15B, lanes 1 and 2). The bands are probably different phosphorylated forms of LT, as reported previously (Livingston et al., 1987). A control cell line, BEn5, was generated by transfecting B104-1-1 cells with pSV2neo and pSV2E (control plasmid similar to pZ189, containing the SV 40 promoter but lacking the LT coding region). As expected, BEn5 and NIH 3T3 cells do not express LT (FIG. 15B, lanes 3 and 4).

Detailed Description Text (163):
These results indicate that liposome-mediated E1A gene transfer can inhibit neu-overexpressing human ovarian cancer cell growth. Therefore, it is predictable that liposome-mediated E1A or LT gene therapy may serve as a powerful therapeutic agent for HER-2 neu-overexpressing human ovarian cancers by direct targeting of E1A or LT at the HER-2 neu-oncogene.

Other Reference Publication (30):
Felgner et al., "Gene Therapeutics: The Direct Delivery of Purified Genes in vivo and Their Application as Drugs, Without the Use of Retroviruses, Is Discussed," Nature, 349:351-352 (1991).

L8: Entry 58 of 62 File: USPT Jun 24, 1997

DOCUMENT-IDENTIFIER: US 5641869 A
TITLE: Method for purifying heregulin

Detailed Description Text (49):
HRG also includes NTD-GFD having its C-terminus at one of the first about 1 to 3 extracellular domain residues (QKR, residues 240-243, HRE-.alpha., FIG. 15) or first about 1-2 transmembrane region residues. In addition, in some HRG-GFD variants the codons are modified at the GFD-transmember proproteolysis site by substitution, insertion or deletion. The GFD proteolysis site is the domain that contains the GFD C-terminal residue and about 5 residues N- and 5 residues C-terminal from this residue. At this time neither the natural C-terminal residue for HRG-.alpha. or HRG-.beta. has been identified, although it is known that Met-227 terminal and Val-229 terminal HRG-.alpha.-GFD are biologically active. The native C-terminus for HRG-.alpha.-GFD is probably Met-227, Lys-228, Val-229, Gln-230, Asn-231 or Gln-232, and for HRG .beta..sub.1 -.beta..sub.2 -GFD is probably Met-226, Ala-227, Ser-228, Phe-229, Trp-230, Lys 231 or (for HRG-.beta..sub.1) K240 or (for HRG-.beta.2) K246. The native C-terminus is determined readily by C-terminal sequencing, although it is not critical that HRG-GFD have the native terminus so long as the GFD sequence possesses the desired activity. In some embodiments of HRG-GFD variants, the amino acid change(s) in the CTP are screened for their ability to resist proteolysis in vitro and inhibit the protease responsible for generation of HRG-GFD.

Detailed Description Text (114):
Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Detailed Description Text (115):
One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1:327,1982), mycophenolic acid (Mulligan et al., Science 209: 1422,1980) or hygromycin (Sugden et al., Mol. Cell. Biol. 5: 410-413,1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G41 8 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Detailed Description Text (117):
For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216, 1980. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR

gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding HRG. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding HRG, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418 (see U.S. Pat. No. 4,965,199).

Detailed Description Text (132):
For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res. 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology 65:499 (1980).

Detailed Description Text (145):
The mammalian host cells used to produce HRG of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58:44 (1979), Barnes and Sato, Anal Biochem., 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985; or U.S. Pat. No. 5,122,469, issued Jun. 16, 1992 Ser. No. 07/592,141, now abandoned filed on 3 Oct. 1990, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin.TM. drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Detailed Description Text (167):
HRG optionally is fused with a polypeptide heterologous to HRG. The heterologous polypeptide optionally is an anchor sequence such as that found in the decay accelerating system (DAF); a toxin such as ricin, pseudomonas exotoxin, gelonin, or other polypeptide that will result in target cell death. These heterologous polypeptides are covalently coupled to HRG through side chains or through the terminal residues. Similarly, HRG is conjugated to other molecules toxic or inhibitory to a target mammalian cell, e.g. such as tricothecenes, or antisense DNA that blocks expression of target genes.

Detailed Description Text (175):
HRG also is entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

Detailed Description Text (202):
Antibodies that are capable of binding to proteolytic processing sites are of particular interest. They are produced either by immunizing with an HRG fragment that includes the CTP processing site, with intact HRG, or with HRG-NTD-GFD and then

screening for the ability to block or inhibit proteolytic processing of HRG into the NTD-GFD fragment by recombinant host cells or isolated cell lines that are otherwise capable of processing HRG to the fragment. These antibodies are useful for suppressing the release of NTD-GFD and therefore are promising for use in preventing the release of NTD-GFD and stimulation of the HER-2 receptor. They also are useful in controlling cell growth and replication. Anti-GFD antibodies are useful for the same reasons, but may not be as efficient biologically as antibodies directed against a processing site.

Detailed Description Text (203):
Antibodies are selected that are capable of binding only to one of the members of the HRG family, e.g. HRG-alpha or any one of the HRG-beta isoforms. Since each of the HRG family members has a distinct GFD-transmembrane domain cleavage site, antibodies directed specifically against these unique sequences will enable the highly specific inhibition of each of the GFDs or processing sites, and thereby refine the desired biological response. For example, breast carcinoma cells which are HER-2 dependent may in fact be activated only by a single GFD isotype or, if not, the activating GFD may originate only from a particular processing sequence, either on the HER-2 bearing cell itself or on a GFD-generating cell. The identification of the target activating GFD or processing site is a straight-forward matter of analyzing HER-2 dependent carcinomas, e.g., by analyzing the tissues for the presence of a particular GFD family member associated with the receptor, or by analyzing the tissues for expression of an HRG family member (which then would serve as the therapeutic target). These selective antibodies are produced in the same fashion as described above, either by immunization with the target sequence or domain, or by selecting from a bank of antibodies having broader specificity.

Detailed Description Text (204):
As described above, the antibodies should have high specificity and affinity for the target sequence. For example, the antibodies directed against GFD sequences should have greater affinity for the GFD than GFD has for the HER-2 receptor. Such antibodies are selected by routine screening methods.

Detailed Description Text (283):
Heregulin-.beta.2 and -.beta.3 variants were isolated in order to obtain cDNA clones that extend further in the 5' direction. A specifically primed cDNA library was constructed in .lambda.gt10 by employing the chemically synthesized antisense primer 3' CCTTCCCGTTCTTCTTCCTCGCTCC (SEQ ID NO:21). This primer is located between nucleotides 167-190 in the sequence of .lambda.her16 (FIG. 4). The isolation of clone .lambda.5'her13 (not to be confused with .lambda.her13) was achieved by hybridizing a synthetic DNA probe corresponding to the 5' end of .alpha.her16 under high stringency conditions with the specifically primed cDNA library. The nucleotide sequence of .lambda.5'her13 is shown in FIG. 11 (SEQ ID NO:22). The 496 base pair nucleotide sequence of .lambda.5'her13 is homologous to the sequence of .lambda.her16 between nucleotides 309-496 of .lambda.5'her13 and 3-190 of .lambda.her16. .lambda.5'her13 extends by 102 amino acids the open reading frame of .lambda.her16.

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L8: Entry 55 of 62 File: USPT Aug 26, 1997

DOCUMENT-IDENTIFIER: US 5660827 A
TITLE: Antibodies that bind to endoglin

Brief Summary Text (5):
Over the past 30 years, fundamental advances in the chemotherapy of neoplastic disease have been realized. While some progress has been made in the development of new chemotherapeutic agents, the more startling achievements have been made in the development of effective regimens for concurrent administration of drugs, and our knowledge of the basic science, e.g., the underlying neoplastic processes at the cellular and tissue level, and the mechanism of action of basic antineoplastic agents. As a result of the fundamental achievement, we can point to significant advances in the chemotherapy of a number of neoplastic diseases, including choriocarcinoma, Wilm's tumor, acute leukemia, rhabdomyosarcoma, retinoblastoma, Hodgkin's disease and Burkitt's lymphoma, to name just a few. Despite the impressive advances that have been made in a few tumors, though, many of the most prevalent forms of human cancer still resist effective chemotherapeutic intervention.

Brief Summary Text (61):
The tumor antigen recognized by the bispecific antibodies employed in the practice of the present invention will be one that is located on the cell surfaces of the tumor being targeted. A large number of solid tumor-associated antigens have now been described in the scientific literature, and the preparation and use of antibodies are well within the skill of the art (see, e.g., Table II hereinbelow). Of course, the tumor antigen that is ultimately selected will depend on the particular tumor to be targeted. Most cell surface tumor targets will only be suitable for imaging purposes, while some will be suitable for therapeutic application. For therapeutic application, preferred tumor antigens will be TAG 72 or the HER-2 proto-oncogene protein, which are selectively found on the surfaces of many breast, lung and colorectal cancers (Thor et al., 1986; Colcher et al., 1987; Shepard et al., 1991). Other targets that will be particularly preferred include milk mucin core protein, human milk fat globule (Miotti et al., 1985; Burchell et al., 1983) and even the high Mr melanoma antigens recognized by the antibody 9.2.27 (Reisfeld et al., 1982).

Detailed Description Text (18):
Such tumor-derived endothelial cell binding factors can be manufactured by the tumor cells themselves, by cells (e.g. macrophages, mast cells) which have infiltrated tumors or by platelets which become activated within the tumor. It is proposed that an antibody or other ligand which recognizes that factor will home selectively to tumor vasculature after injection. Such an antibody or ligand should thus enable the imaging or targeting of drugs or other agents to solid tumors. Further, such an antibody may be specific for a factor/factor receptor complex present on the surface of the tumor vasculature, so that the antibody recognizes only a factor/factor receptor complex, while not binding to either the factor or the factor receptor individually.

Detailed Description Text (36):
A strategy for confining Class II expression to tumor vasculature is to suppress IFN-.gamma. production through out the animal by administering CsA and then to induce IFN-.gamma. production specifically in the tumor by targeting a CsA-resistant T cell activator to the tumor. A bispecific (Fab'--Fab') antibody having one arm directed against a tumor antigen and the other arm directed against CD28 should

localize in the tumor and then crosslink CD28 antigens on T cells in the tumor. Crosslinking of CD28, combined with a second signal (provided, for example, by IL-1 which, is commonly secreted by tumor cells (Burrows et al., 1991; Ruco et al., 1990) has been shown to activate T cells through a CA2+-independent non-CsA-inhibitable pathway (Hess et al., 1991; June et al., 1987; Bjorndahl et al., 1989). The T cells that should be activated in the tumor are those adjacent to the vasculature since this is the region most accessible to cells and is also where the bispecific antibody will be most concentrated. The activated T cells should then secrete IFN-.gamma. which induces Class II antigens on the adjacent tumor vasculature.

Detailed Description Text (38):
Secondly, hepatic Kupffer cells and probably other cells of monocyte/macrophage lineage are not killed by the anti-Class II immunotoxin even though it binds to them. No morphological changes in the Kupffer cells are visible even several days after administration of the immunotoxin. This is probably because cells of monocyte/macrophage linage are generally resistant to immunotoxin-mediated killing (Engert et al., 1991). Cells of monocyte/macrophage lineage appear to bind and internalize immunotoxins but route them to the lysosomes where they are destroyed, unlike other cell types which route immunotoxins to the trans-Golgi region or the E.R. which are thought to be 10 site(s) from which ricin A chain enters the cytosol (Van Deurs et al., 1986; Van Deurs et al., 1988).

Detailed Description Text (61):
The spacer arm between these two reactive groups of any cross-linkers may have various length and chemical composition. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (e.g., benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (e.g., disulfide bond resistant to reducing agents).

Detailed Description Text (76):
In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc. For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express constructs encoding the targeting agent/toxin compounds may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with targeting agent/toxin DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

Detailed Description Text (77):
A number of selection systems may be used, including, but not limited, to the herpes simplex virus thymidine kinase (Wigler et al., 1977), hypoxanthineoguanine phosphoriboxyltransferase (Szybalska et ai., 1962), and adenine phsophoribosyltransferase genes (Lowy et al., 1980) can be employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980; O'Hare et al., 1981); gpt, which confers resistance to mycophenolic acid (Mulligan et al., 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981); and hygro, which confers resistance to hygromycin

(Santerre et al., 1984).

Detailed Description Text (82):
It is contemplated that most therapeutic applications of the present invention will involve the targeting of a toxin moiety to the tumor endothelium. This is due to the much greater ability of most toxins to deliver a cell killing effect as compared to other potential agents. However, there may be circumstances, such as when the target antigen does not internalize by a route consistent with efficient intoxication by targeting agent/toxin compounds, such as immunotoxins, where one will desire to target chemotherapeutic agents such as antitumor drugs, other cytokines, antimetabolites, alkylating agents, hormones, and the like. The advantages of these agents over their non-targeting agent conjugated counterparts is the added selectivity afforded by the targeting agent, such as an antibody. One might mention by way of example agents such as steroids, cytosine arabinoside, methotrexate, aminopterin, anthracyclines, mitomycin C, vinca alkaloids, demecolcine, etoposide, mithramycin, and the like. This list is, of course, merely exemplary in that the technology for attaching pharmaceutical agents to targeting agents, such as antibodies, for specific delivery to tissues is well established (see, e.g., Ghose & Blair, 1987).

Detailed Description Text (86):
Thus, it is generally believed to be possible to conjugate to antibodies any pharmacologic agent that has a primary or secondary amine group, hydrazide or hydrazine group, carboxyl alcohol, phosphate, or alkylating group available for binding or cross-linking to the amino acids or carbohydrate groups of the antibody. In the case of protein structures, this is most readily achieved by means of a cross linking agent (see preceding section on immunotoxins). In the case of doxorubicin and daunomycin, attachment may be achieved by means of an acid labile acyl hydrazone or cis aconityl linkage between the drug and the antibody. Finally, in the case of methotrexate or aminopterin, attachment is achieved through a peptide spacer such as L--Leu--L--Ala--L--Leu--L--Ala, between the .gamma.-carboxyl group of the drug and an amino acid of the antibody. For a general overview of linking technology, one may wish to refer to Ghose & Blair (1987).

Detailed Description Text (142):
Antibiotic treatment had no effect on Ia.sup.d expression by tumor endothelial cells. In subsequent studies it was found that SCID mice had little Ia.sup.d on colonic epithelial or endothelial cells and that intravenously-administered anti-Ia.sup.d antibody did not localize to their colonic endothelium. Furthermore, high doses of M5/114 immunotoxins were non-toxic in these animals. Given the possibility of antibiotic resistance arising in the gut flora of tetracycline-treated BALB/c nu/nu mice, we believe that SCID mice may be more suitable for these types of studies.

Detailed Description Text (182):
MHC Class II antigens are also expressed by B-lymphocytes, some bone marrow cells, myeloid cells and some renal and gut epithelia in BALB/c nu/nu mice, however, therapeutic doses of anti-Class II immunotoxin did not cause any permanent damage to these cell populations. Splenic B cells and bone marrow myelocytes bound intravenously injected anti-Class II antibody but early bone marrow progenitors do not express Class II antigens and mature bone marrow subsets and splenic B cell compartments were normal 3 weeks after therapy, so it is likely that any Ia.sup.+ myelocytes and B cells killed by the immunotoxin were replaced from the stem cell pool. It is contemplated that the existence of large numbers of readily accessible B cells in the spleen prevented the anti-Class II immunotoxin from reaching the relatively inaccessible Ia.sup.+ epithelial cells but hepatic Kupffer cells were not apparently damaged by M5/114-dgA despite binding the immunotoxin. Myeloid cells are resistant to ricin A-chain immunotoxins, probably due to unique endocytic pathways related to their degradative physiologic function (Engert et al., 1991).

Detailed Description Text (192):
C3H/He mice will be injected subcutaneously with syngeneic MM102 mammary tumor cells. The tumor cells express Ly6.2 which is a unique marker in C3H mice (Ly6.1 positive). Mice bearing solid MM102 mammary tumors will be treated with CsA to reduce or abolish Class II expression throughout the vasculature. As originally

shown in the dog (Groenewegen et al., 1985), and, as recently confirmed by the inventors in the mouse, CsA inhibits T cell and NK cell activation and lowers the basal levels of IFN-.gamma. to the extent that Class II disappears from the vasculature. The mice will then be injected with a bispecific (Fab'--Fab') anti-CD28/anti-Ly6A.2 antibody, which should localize to the tumor by virtue of its Ly6.2-binding activity. The bispecific antibody should then bind to T cells which are present in (or which subsequently infiltrate (Blanchard et al., 1988) the tumor. Crosslinking of CD28 antigens on the T cells by multiple molecules of bispecific antibody attached to the tumor cells should activate the T cells via the CsA-resistant CD28 pathway (Hess et al., 1991; June et al., 1987; Bjorndahl et al., 1989). Activation of T cells should not occur elsewhere because the crosslinking of CD28 antigens which is necessary for activation (Thompson et al., 1989; Koulova et al., 1991) should not occur with soluble, non-tumor cell bound, bispecific antibody. T cells which become activated in the tumor should release IFN-.gamma. which should induce Class II antigens on the tumor vascular endothelium (Collins et al., 1984; Pober et al., 1983) and probably on the tumor cells themselves (Boyer et al., 1989). Animals will then be treated with anti-Class II immunotoxins to destroy the tumor blood supply.

Detailed Description Text (212):
It will be important to confirm that tumor cells coated with the bispecific antibody, but not free bispecific antibody, are able to activate T cells in a CsA-resistant fashion. T cells will be enriched from the spleens of C3H/He mice by depleting B-cells and macrophages according to the procedure of Lee and colleagues 1990 (Lee, et al., 1990). Spleen cells are treated with mouse anti-Class II antibody and the Class II-expressing cells are removed by treating them with goat anti-mouse IgG-coupled magnetic beads and withdrawing them with a strong magnet. The non-adherent cells are decanted and are treated further to remove residual B cells and macrophages by successive rounds of treatment with anti-J11D plus BRC and anti-MAC-1 antibody plus goat anti-rat serum. After these procedures, the remaining cells are .gtoreq.95% T cells and <3% Ig positive.

Detailed Description Text (238):
C3H/He mice bearing 1.0-1.3 cm MM102 or MM48 tumors will be injected i.v with anti-Ly6A.2/anti-CD14 bispecific antibody or with various control materials including unconjugated anti-Ly6A.2 and anti-CD14 antibodies (Fab' and IgG) and diluent alone. Tumors will be removed at various times and cryostat sections will be cut and stained with rat monoclonal antibodies to murine ELAM-1, using standard indirect immunoperoxidase techniques. The presence of the bispecific antibody on tumor cells will be verified by staining for rat immunoglobulin. Resident macrophages and infiltrating monocytes will be detected by indirect immunoperoxidase staining with anti-Mac-1 (CD 11b/CD 18) antibodies. Cytokine-producing cells will be identified in serial cryostat sections of tumors by in situ hybridization with .sup.35 S-labeled antisense asymmetric RNA probes for murine IL-1.beta. and TNF.alpha. mRNA.

Detailed Description Text (333):
Thirdly, the antibodies could be used for therapy. The highly accessible location of endoglin on the luminal surface of the tumor vasculature is especially advantageous for therapeutic application, because all of the target endothelial cells are able to bind the therapeutic antibody, as shown in Example I. Both TEC-4 and TEC-11 are complement-fixing and so might induce selective lysis of endothelial cells in the tumor vascular bed. Also, the antibodies could be used to deliver therapeutic quantities of radioisotopes, toxins, chemotherapeutic drugs or coagulants to the tumor vasculature. Animal studies indicates that anti-tumor endothelial cell immunotoxins are most effective when combined with anti-tumor cell immunotoxins, which kill those tumor cells that have invaded surrounding normal host tissue (as disclosed hereinabove and in Burrows and Thorpe, 1993). Thus, TEC-4 or TEC-11 could be used clinically in combination with antibodies against well-characterized tumor markers such as p185.sup.HER-2, TAG-72, and CO17-1A (Shepard et al., 1991; Greiner et al., 1991; Kaplan, 1989) or indeed with conventional chemotherapeutic drugs.

Detailed Description Text (443):
Ghose, et al. (1987) CRC Critical Reviews in Therapeutic Drug Carrier Systems, 3:262-359.

L8: Entry 53 of 62 File: USPT Jun 30, 1998

DOCUMENT-IDENTIFIER: US 5773476 A
TITLE: Methods and compositions for inhibiting cell proliferative disorders

Brief Summary Text (10):
Examples of specific receptor tyrosine kinases associated with cell proliferative disorders include, platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and HER2. The gene encoding HER2 (her-2) is also referred to as neu, and c-erbB-2 (Slamon, D. J., et al., Science, 235:177-182, 1987).

Brief Summary Text (22):
We find that these compounds are potent blockers of EGFR kinase and its homolog HER-2 kinase.

Brief Summary Text (23):
Interestingly, we find that certain S-aryltyrphostins discriminate between EGFR and HER-2 kinase in favor of the HER-2 kinase domain by almost 2 orders of magnitude. When examined in intact cells it was found that these selective S-aryltyrphostins are equipotent in inhibiting EGF dependent proliferation of NIH 3T3 harboring either the EGF receptor or the chimera EGF/neu HER1-2.

Brief Summary Text (33):
When used as a therapeutic the compounds described herein are preferably administered with a physiologically acceptable carrier. A physiologically acceptable carrier is a formulation to which the compound can be added to dissolve it or otherwise facilitate its administration. Examples of physiologically acceptable carriers include water, saline, physiologically buffered saline, cyclodextrins and PBTE:D5W (described below). Hydrophobic compounds are preferably administered using a carrier such as PBTE:D5W. An important factor in choosing an appropriate physiologically acceptable carrier is choosing a carrier in which the compound remains active or the combination of the carrier and the compound produces an active compound. The compound may also be administered in a continuous fashion using a slow release formulation or a pump to maintain a constant or varying drug level in a patient.

Detailed Description Text (87):
Examples of cell lines which can be used to study the effect of a compound, for example in vitro or in animal models, include the following: cells characterized by over-activity of HER2 include SKOV3 (ATCC# HTB77), Calu3 (ATCC# HTB25), MVA361 (ATCC# HTB27), and SW626 (ATCC# HTB78) ; cell lines characterized by inappropriate activity of PDGFR such as human glioblastoma cell line T98G; and cell lines characterized by inappropriate activity of EGFR such as A431 (ATTC# CRL1555) and KB (ATTC# CCL17). One skilled in the art can choose other suitable cell lines using standard techniques and the present application as a guide. For example, the diagnostic section described infra can be used to help determine whether a cell line (e.g., a tumor cell line) is driven by a tyrosine receptor kinase such as HER-2.

Detailed Description Text (91):
To study the effect of anti-tumor drug candidates on HER2 expressing tumors, the tumor cells should be able to grow in the absence of supplemental estrogen. Many mammary cell lines are dependent on estrogen for in vivo growth in nude mice (Osborne et al., supra), however, exogenous estrogen suppresses her2 expression in nude mice (Warri et al., supra, Dati, C., et al, Oncogene, 5:1001-1006, 1990). For

example, in the presence of estrogen, MCF-7, ZR-75-1, and T47D cells grow well in vivo, but express very low levels of HER2 (Warri et al., supra, Dati, C., et al, Oncogene, 5:1001-1006).

Detailed Description Text (97):
In addition to measuring tumor growth to achieve a compound range which can safely be administered to a patient in the animal models, plasma half-life and biodistribution of the drug and metabolites in plasma, tumors, and major organs can be determined to facilitate the selection of drugs most appropriate for the inhibition of a disorder. Such measurements can be carried out, for example, using HPLC analysis. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as model.

Detailed Description Text (107):
HER2 driven disorders are characterized by inappropriate or over-activity of HER2. Inappropriate HER-2 activity refers to either: 1) HER2 expression in cells which normally do not express HER2; 2) increased HER-2 expression leading to unwanted cell proliferation such as cancer; 3) increased HER-2 activity leading to unwanted cell proliferation, such as cancer; and/or over-activity of HER-2.

Detailed Description Text (109):
The HER-2 protein is a member of the class I receptor tyrosine kinase (RTK) family. Yarden and Ullrich, Annu. Rev. Biochem. 57:443, 1988; Ullrich and Schlessinger, Cell 61:203, 1990. HER-2 protein is structurally related to EGF-R, p180(HER-3), and p180(HER-4). Carraway, et al ., Cell 78:5, 1994; Carraway, et al., J. Biol. Chem. 269:14303, 1994. These receptors share a common molecular architecture and contain two cysteine-rich regions within their cytoplasmic domains and structurally related enzymatic regions within their cytoplasmic domains.

Detailed Description Text (110):
Activation of HER-2 protein can be caused by different events such as ligand-stimulated homo-dimerization, ligand-stimulated hetero-dimerization and ligand-independent homo-dimerization. Ligand-stimulated hetero-dimerization appears to be induced by EGF-R to form EGF-R/HER-2 complexes and by neu differentiation factor/heregulin (NDF/HRG) to form HER-2/ HER-3 and/or HER-2/ HER-4 complexes. Wada et al., Cell 61:1339, 1990; Slikowski et al., J. Biol. Chem. 269:14661, 1994; Plowman et al., Nature 266:473, 1993. Ligand-dependent activation of HER-2 protein is thought to be mediated by neu-activating factor (NAF) which can directly bind to p185(HER-2) and stimulate enzymatic activity. Dougall et al., Oncogene 9:2109, 1994; Samata et al., Proc. Natl. Acad. Sci. USA 91:1711, 1994. Ligand-independent homo-dimerization of HER-2 protein and resulting receptor activation is facilitated by over-expression of HER-2 protein.

Detailed Description Text (112):
HER-2 protein substrates are acted upon by activated HER-2 complexes such as HER-2/EGF-R, HER-2/HER-2, HER-2/ HER-3, and HER-2/HER-4 activated complexes. An activated HER-2 complex acts as a phosphokinase and phosphorylates different cytoplasmic proteins. Examples of HER-2 substrates include, IP.sub.3 kinase and PI 4-kinase. Scott et al., Journal of Biological Chemistry 22:14300, 1991.

Detailed Description Text (113):
HER-2 adaptor proteins bind to an activated HER-2 complex and then another protein. For example, GRB-7 binding to a HER-2 complex may be sufficient to initiate the GRB-7 signaling pathway without phosphorylation. Stein et al., EMBO Journal 13:1331, 1993.

Detailed Description Text (114):
Thus, HER-2 protein activities include: (1) phosphorylation of HER-2 protein, HER-3 protein or HER-4 protein; (2) phosphorylation of a HER-2 protein substrate; (3) interaction with a HER-2 adapter protein; and/or (4) HER-2 protein surface expression. Additional HER-2 protein activities can be identified using standard techniques. For example, a partial agonistic monoclonal antibody recognizing HER-2 protein can be used to activate HER-2 protein and examine signal transduction of

HER-2 protein. Scott et al., Journal of Biological Chemistry 22:14300, 1991.

Detailed Description Text (142):
The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used, and the size and physiological condition of the patient. For the treatment of cancers the expected daily dose is between 1 to 2000 mg/day, preferably 1 to 250 mg/day, and most preferably 10 to 150 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.

Detailed Description Text (143):
A factor which can influence the drug dose is body weight. Drugs should be administered at doses ranging from 0.02 to 25 mg/kg/day, preferably 0.02 to 15 mg/kg/-day, most preferably 0.2 to 15 mg/kg/day. Alternatively, drugs can be administered at 0.5 to 1200 mg/m.sup.2 /day, preferably 0.5 to 150 mg/m.sup.2 /day, most preferably 5 to 100 mg/m.sup.2 /day. The average plasma level should be 50 to 5000 .mu.g/ml, preferably 50 to 1000 .mu.g/ml, and most preferably 100 to 500 .mu.g/ml. Plasma levels may be reduced if pharmacological effective concentrations of the drug are achieved at the site of interest.

Detailed Description Text (145):
The receptor tyrosine kinase inhibitory compounds described herein can be used alone, in combination with other agents able to inhibit protein kinase activity (e.g., anti-sense nucleic acid and ribozymes targeted to nucleic acid encoding a receptor tyrosine kinase, and antibodies able to modulate tyrosine kinase activity, such as anti-HER-2 antibodies which may work by modulating HER-2 activity as described by Hudziak et al., Mol. Cell. Biol. 9:1165, 1989; Sarup et al., Growth Regulation 1:71, 1991; and Shepard et al., J. clinical Immunology 11:117, 1991) and in combination with other types of treatment for cell proliferative disorders.

Detailed Description Text (155):
Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention. Rather, they illustrate methodology by which drugs having the disclosed formulas can be readily identified by routine procedure to ensure that they have the desired activity, and the synthesis of different compounds described herein. Compounds within a formula claimed herein can be screened to determine those with the most appropriate activity prior to administration to an animal or human. Other compounds can also be screened to determine suitability for use in methods of this invention.

Detailed Description Text (159):
SKBR3 cells over-express HER2. A431 cells over-express EGFR. These cells were dispensed into 96-well plates with test compounds. After 4 days the monolayers were TCA-fixed then stained with sulphorhodamine B. The absorbance versus log drug concentration was plotted and IC.sub.50 values estimated.

Detailed Description Text (184):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for one hour.

Detailed Description Text (187):
D. After two hours incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (188):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TEST as described above.

Detailed Description Text (216):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug

stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for two hours.

Detailed Description Text (220):
D. After 120 minutes incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (221):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

Detailed Description Text (256):
(a) Test the drugs in serum-free condition. Before adding drugs, the old media is replaced with serum-free RPMI (90 .mu.l/well).

Detailed Description Text (257):
(b) Dilute drug stock (in 100% DMSO) 1:10 with RPMI, and transfer 10 .mu.l/well of this solution to the cells to achieve a final drug DMSO concentration at 1%. Incubate the cells in 5% CO.sub.2 at 37.degree. C.

Detailed Description Text (259):
(d) After drug preincubation for two hours remove all the solution from the plate, transfer HNTG* 100 .mu.l/well to the cells, and shake for 10 minutes.

Detailed Description Text (321):
8. MCF-7/ HER-2 SRB Growth Assay

Other Reference Publication (1):
G. Kaur, A. Gazit, A. Levitzki, E. Stowe, D. Cooney, E. Sausville, Anti-Cancer Drugs 1994, vol. 5, pp. 213-222, Mar. 1994.

Other Reference Publication (40):
Schornagel et al., "Synthesis and Evaluation of 2,4-Diaminoquinazoline Antifolates with Activity Against Methotrexate-Resistant Human Tumor Cells," Biochem. Pharm. 33(20):3251-3255 (1984).

Other Reference Publication (45):
Skehan et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," J. Natl. Cancer Inst. 82:1107-1112 (1990).

CLAIMS:

3. The compound of claim 1, wherein said compound inhibits HER-2 activity.

7. The compound of claim 4, wherein said compound inhibits HER-2 activity.

L8: Entry 53 of 62 File: USPT Jun 30, 1998

DOCUMENT-IDENTIFIER: US 5773476 A
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HER-2 protein. Scott et al., Journal of Biological Chemistry 22:14300, 1991.

Detailed Description Text (142):
The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used, and the size and physiological condition of the patient. For the treatment of cancers the expected daily dose is between 1 to 2000 mg/day, preferably 1 to 250 mg/day, and most preferably 10 to 150 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.

Detailed Description Text (143):
A factor which can influence the drug dose is body weight. Drugs should be administered at doses ranging from 0.02 to 25 mg/kg/day, preferably 0.02 to 15 mg/kg/-day, most preferably 0.2 to 15 mg/kg/day. Alternatively, drugs can be administered at 0.5 to 1200 mg/m.sup.2 /day, preferably 0.5 to 150 mg/m.sup.2 /day, most preferably 5 to 100 mg/m.sup.2 /day. The average plasma level should be 50 to 5000 .mu.g/ml, preferably 50 to 1000 .mu.g/ml, and most preferably 100 to 500 .mu.g/ml. Plasma levels may be reduced if pharmacological effective concentrations of the drug are achieved at the site of interest.

Detailed Description Text (145):
The receptor tyrosine kinase inhibitory compounds described herein can be used alone, in combination with other agents able to inhibit protein kinase activity (e.g., anti-sense nucleic acid and ribozymes targeted to nucleic acid encoding a receptor tyrosine kinase, and antibodies able to modulate tyrosine kinase activity, such as anti-HER-2 antibodies which may work by modulating HER-2 activity as described by Hudziak et al., Mol. Cell. Biol. 9:1165, 1989; Sarup et al., Growth Regulation 1:71, 1991; and Shepard et al., J. clinical Immunology 11:117, 1991) and in combination with other types of treatment for cell proliferative disorders.

Detailed Description Text (155):
Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention. Rather, they illustrate methodology by which drugs having the disclosed formulas can be readily identified by routine procedure to ensure that they have the desired activity, and the synthesis of different compounds described herein. Compounds within a formula claimed herein can be screened to determine those with the most appropriate activity prior to administration to an animal or human. Other compounds can also be screened to determine suitability for use in methods of this invention.

Detailed Description Text (159):
SKBR3 cells over-express HER2. A431 cells over-express EGFR. These cells were dispensed into 96-well plates with test compounds. After 4 days the monolayers were TCA-fixed then stained with sulphorhodamine B. The absorbance versus log drug concentration was plotted and IC.sub.50 values estimated.

Detailed Description Text (184):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for one hour.

Detailed Description Text (187):
D. After two hours incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (188):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TEST as described above.

Detailed Description Text (216):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug

stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for two hours.

Detailed Description Text (220):
D. After 120 minutes incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (221):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

Detailed Description Text (256):
(a) Test the drugs in serum-free condition. Before adding drugs, the old media is replaced with serum-free RPMI (90 .mu.l/well).

Detailed Description Text (257):
(b) Dilute drug stock (in 100% DMSO) 1:10 with RPMI, and transfer 10 .mu.l/well of this solution to the cells to achieve a final drug DMSO concentration at 1%. Incubate the cells in 5% CO.sub.2 at 37.degree. C.

Detailed Description Text (259):
(d) After drug preincubation for two hours remove all the solution from the plate, transfer HNTG* 100 .mu.l/well to the cells, and shake for 10 minutes.

Detailed Description Text (321):
8. MCF-7/ HER-2 SRB Growth Assay

Other Reference Publication (1):
G. Kaur, A. Gazit, A. Levitzki, E. Stowe, D. Cooney, E. Sausville, Anti-Cancer Drugs 1994, vol. 5, pp. 213-222, Mar. 1994.

Other Reference Publication (40):
Schornagel et al., "Synthesis and Evaluation of 2,4-Diaminoquinazoline Antifolates with Activity Against Methotrexate-Resistant Human Tumor Cells," Biochem. Pharm. 33(20):3251-3255 (1984).

Other Reference Publication (45):
Skehan et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," J. Natl. Cancer Inst. 82:1107-1112 (1990).

CLAIMS:

3. The compound of claim 1, wherein said compound inhibits HER-2 activity.

7. The compound of claim 4, wherein said compound inhibits HER-2 activity.

L8: Entry 51 of 62 File: USPT Aug 4, 1998

DOCUMENT-IDENTIFIER: US 5789427 A
TITLE: Methods and compositions for inhibiting cell proliferative disorders

Brief Summary Text (10):
Examples of specific receptor tyrosine kinases associated with cell proliferative disorders include, platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and HER2. The gene encoding HER2 (her-2) is also referred to as neu, and cerbB-2 (Slamon, D. J., et al., Science, 235:177-182, 1987).

Brief Summary Text (23):
We find that these compounds are potent blockers of EGFR kinase and its homolog HER-2 kinase. Interestingly, we find that certain S-aryltyrphostins discriminate between EGFR and HER-2 kinase in favor of the HER-2 kinase domain by almost 2 orders of magnitude. When examined in intact cells it was found that these selective S-aryltyrphostins are equipotent in inhibiting EGF dependent proliferation of NIH 3T3 harboring either the EGF receptor or the chimera EGF/neu HER1-2.

Brief Summary Text (32):
When used as a therapeutic the compounds described herein are preferably administered with a physiologically acceptable carrier. A physiologically acceptable carrier is a formulation to which the compound can be added to dissolve it or otherwise facilitate its administration. Examples of physiologically acceptable carriers include water, saline, physiologically buffered saline, cyclodextrins and PBTE:D5W (described below). Hydrophobic compounds are preferably administered using a carrier such as PBTE:D5W. An important factor in choosing an appropriate physiologically acceptable carrier is choosing a carrier in which the compound remains active or the combination of the carrier and the compound produces an active compound. The compound may also be administered in a continuous fashion using a slow release formulation or a pump to maintain a constant or varying drug level in a patient.

Detailed Description Text (87):
Examples of cell lines which can be used to study the effect of a compound, for example in vitro or in animal models, include the following: cells characterized by over-activity of HER2 include SKOV3 (ATCC# HTB77), Calu3 (ATCC# HTB25), MVA361 (ATCC# HTB27), and SW626 (ATCC# HTB78); cell lines characterized by inappropriate activity of PDGFR such as human glioblastoma cell line T98G; and cell lines characterized by inappropriate activity of EGFR such as A431 (ATTC# CRL1555) and KB (ATTC# CCL17). One skilled in the art can choose other suitable cell lines using standard techniques and the present application as a guide. For example, the diagnostic section described infra can be used to help determine whether a cell line (e.g., a tumor cell line) is driven by a tyrosine receptor kinase such as HER-2.

Detailed Description Text (91):
To study the effect of anti-tumor drug candidates on HER2 expressing tumors, the tumor cells should be able to grow in the absence of supplemental estrogen. Many mammary cell lines are dependent on estrogen for in vivo growth in nude mice (Osborne et al., supra), however, exogenous estrogen suppresses her2 expression in nude mice (Warri et al., supra, Dati, C., et al, Oncogene, 5:1001-1006, 1990). For example, in the presence of estrogen, MCF-7, ZR-75-1, and T47D cells grow well in vivo, but express very low levels of HER2 (Warri et al., supra, Dati, C., et al, Oncogene, 5:1001-1006).

Detailed Description Text (97):
In addition to measuring tumor growth to achieve a compound range which can safely be administered to a patient in the animal models, plasma half-life and biodistribution of the drug and metabolites in plasma, tumors, and major organs can be determined to facilitate the selection of drugs most appropriate for the inhibition of a disorder. Such measurements can be carried out, for example, using HPLC analysis. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as model.

Detailed Description Text (107):
HER2 driven disorders are characterized by inappropriate or over-activity of HER2. Inappropriate HER-2 activity refers to either: 1) HER2 expression in cells which normally do not express HER2; 2) increased HER-2 expression leading to unwanted cell proliferation such as cancer; 3) increased HER-2 activity leading to unwanted cell proliferation, such as cancer; and/or over-activity of HER-2.

Detailed Description Text (109):
The HER-2 protein is a member of the class I receptor tyrosine kinase (RTK) family. Yarden and Ullrich, Annu. Rev. Biochem. 57:443, 1988; Ullrich and Schlessinger, Cell 61:203, 1990. HER-2 protein is structurally related to EGF-R, p180(HER-3), and p180(HER-4). Carraway, et al., Cell 78:5, 1994; Carraway, et al., J. Biol. Chem. 269:14303, 1994. These receptors share a common molecular architecture and contain two cysteine-rich regions within their cytoplasmic domains and structurally related enzymatic regions within their cytoplasmic domains.

Detailed Description Text (110):
Activation of HER-2 protein can be caused by different events such as ligand-stimulated homodimerization, ligand-stimulated hetero-dimerization and ligand-independent homo-dimerization. Ligand-stimulated hetero-dimerization appears to be induced by EGF-R to form EGF-R/HER-2 complexes and by neu differentiation factor/heregulin (NDF/HRG) to form HER-2/HER-3 and/or HER2/HER-4 complexes. Wada et al., Cell 61:1339, 1990; Slikowski et al., J. Biol. Chem. 269:14661, 1994; Plowman et al., Nature 266:473, 1993. Ligand-dependent activation of HER-2 protein is thought to be mediated by neuactivating factor (NAF) which can directly bind to p185(HER-2) and stimulate enzymatic activity. Dougall et al., Oncogene 9:2109, 1994; Samata et al., Proc. Natl. Acad. Sci. USA 91:1711, 1994. Ligand-independent homodimerization of HER-2 protein and resulting receptor activation is facilitated by over-expression of HER-2 protein.

Detailed Description Text (112):
HER-2 protein substrates are acted upon by activated HER-2 complexes such as HER-2/EGF-R, HER-2/HER-2, HER2/HER-3, and HER-2/HER-4 activated complexes. An activated HER-2 complex acts as a phosphokinase and phosphorylates different cytoplasmic proteins. Examples of HER-2 substrates include, IP.sub.3 kinase and PI 4-kinase. Scott et al., Journal of Biological Chemistry 22:14300, 1991.

Detailed Description Text (113):
HER-2 adaptor proteins bind to an activated HER-2 complex and then another protein. For example, GRB-7 binding to a HER-2 complex may be sufficient to initiate the GRB-7 signaling pathway without phosphorylation. Stein et al., EMBO Journal 13:1331, 1993.

Detailed Description Text (114):
Thus, HER-2 protein activities include: (1) phosphorylation of HER-2 protein, HER-3 protein or HER-4 protein; (2) phosphorylation of a HER-2 protein substrate; (3) interaction with a HER-2 adapter protein; and/or (4) HER-2 protein surface expression. Additional HER-2 protein activities can be identified using standard techniques. For example, a partial agonistic monoclonal antibody recognizing HER-2 protein can be used to activate HER-2 protein and examine signal transduction of HER-2 protein. Scott et al., Journal of Biological Chemistry 22:14300, 1991.

Detailed Description Text (141):

The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used, and the size and physiological condition of the patient. For the treatment of cancers the expected daily dose is between 1 to 2000 mg/day, preferably 1 to 250 mg/day, and most preferably 10 to 150 mg/day. Drugs can be delivered less frequently provided plasma levels of the active moiety are sufficient to maintain therapeutic effectiveness.

Detailed Description Text (142):
A factor which can influence the drug dose is body weight. Drugs should be administered at doses ranging from 0.02 to 25 mg/kg/day, preferably 0.02 to 15 mg/kg/day, most preferably 0.2 to 15 mg/kg/day. Alternatively, drugs can be administered at 0.5 to 1200 mg/m.sup.2 /day, preferably 0.5 to 150 mg/m.sup.2 /day, most preferably 5 to 100 mg/m.sup.2 /day. The average plasma level should be 50 to 5000 .mu.g/ml, preferably 50 to 1000 .mu.g/ml, and most preferably 100 to 500 .mu.g/ml. Plasma levels may be reduced if pharmacological effective concentrations of the drug are achieved at the site of interest.

Detailed Description Text (144):
The receptor tyrosine kinase inhibitory compounds described herein can be used alone, in combination with other agents able to inhibit protein kinase activity (e.g., anti-sense nucleic acid and ribozymes targeted to nucleic acid encoding a receptor tyrosine kinase, and antibodies able to modulate tyrosine kinase activity, such as anti-HER-2 antibodies which may work by modulating HER2 activity as described by Hudziak et al., Mol. Cell. Biol. 9:1165, 1989; Sarup et al., Growth Regulation 1:71, 1991; and Shepard et al., J. clinical Immunology 11:117, 1991) and in combination with other types of treatment for cell proliferative disorders.

Detailed Description Text (154):
Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention. Rather, they illustrate methodology by which drugs having the disclosed formulas can be readily identified by routine procedure to ensure that they have the desired activity, and the synthesis of different compounds described herein. Compounds within a formula claimed herein can be screened to determine those with the most appropriate activity prior to administration to an animal or human. Other compounds can also be screened to determine suitability for use in methods of this invention.

Detailed Description Text (158):
SKBR3 cells over-express HER2. A431 cells over-express EGFR. These cells were dispensed into 96-well plates with test compounds. After 4 days the monolayers were TCA-fixed then stained with sulphorhodamine B. The absorbance versus log drug concentration was plotted and IC50 values estimated.

Detailed Description Text (181):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for one hour.

Detailed Description Text (184):
D. After two hours incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (185):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

Detailed Description Text (212):
A. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 .mu.l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO.sub.2 at 37.degree. C. for two hours.

Detailed Description Text (215):
D. After 120 minutes incubation with drug, add prepared EGF ligand to cells, 10 .mu.l per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

Detailed Description Text (216):
E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 .mu.l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

Detailed Description Text (250):
(a) Test the drugs in serum-free condition. Before adding drugs, the old media is replaced with serum-free RPMI (90 .mu.l/well).

Detailed Description Text (251):
(b) Dilute drug stock (in 100% DMSO) 1:10 with RPMI, and transfer 10 .mu.l/well of this solution to the cells to achieve a final drug DMSO concentration at 1%. Incubate the cells in 5% CO.sub.2 at 37.degree. C.

Detailed Description Text (253):
(d) After drug preincubation for two hours remove all the solution from the plate, transfer HNTG* 100 .mu.l/well to the cells, and shake for 10 minutes.

Other Reference Publication (59):
Schornagel et al., "Synthesis and Evaluation of 2,4-Diaminoquinazoline Antifolates with Activity Against Methotrexate-Resistant Human Tumor Cells," Biochem, Pharm. 33(200:3251-3255 (1984).

Other Reference Publication (64):
Skehan et al., "New Colormetric Cytotoxicity Assay for Anticancer-Drug Screening," J. Natl. Cancer Inst. 82:1107-1112 (1990).

L8: Entry 49 of 62 File: USPT Sep 15, 1998

DOCUMENT-IDENTIFIER: US 5808036 A
TITLE: Stem-loop oligonucleotides containing parallel and antiparallel binding domains

Brief Summary Text (2):
The present invention provides stem-loop oligonucleotides capable of strong binding to a target DNA or RNA. Moreover, stem-loop oligonucleotides are resistant to nucleases and bind to a target with high selectivity and affinity. Such strong binding allows the present stem-loop oligonucleotides to be utilized in a variety of ways. For example, stem-loop oligonucleotides can be labeled for use as probes to detect or isolate a target nucleic acid. Stem-loop oligonucleotides can also be transcribed within, or administered to, a cell to provide in vivo regulators of DNA replication, RNA transcription, protein translation, reverse transcription, and other processes involving nucleic acid templates.

Brief Summary Text (7):
Furthermore, there has been great interest recently in developing oligonucleotides as regulators of cellular nucleic acid biological function. This interest arises from observations on naturally occurring complementary, or antisense, RNA used by some cells to control protein expression. However, the development of oligonucleotides for in vivo regulation of biological processes has been hampered by several long-standing problems, including the low binding affinity and nuclease sensitivity of linear oligonucleotides.

Brief Summary Text (9):
Splicing of a pre-mRNA transcript essential for Herpes Simplex virus replication has also been inhibited with a linear oligonucleotide which was complementary to an acceptor splice junction. In this instance, a methylphosphonate linkage was employed in the linear oligonucleotide to increase its nuclease resistance. Addition of this chemically-modified oligonucleotide to the growth medium caused reduction in protein synthesis and growth of uninfected cells, most likely because of toxicity problems occurring at high oligonucleotide concentrations (Smith et al., 1986, Proc. Natl. Acad. Sci. USA 83:2787-2791).

Brief Summary Text (16):
The present invention provides stem-loop oligonucleotides which have many of the desirable attributes of circular oligonucleotides, e.g. nuclease resistance (Tang et al., 1993, Nucleic Acids Res. 21:2729-2735). However the present stem-loop oligonucleotides are much simpler to make both in vivo and in vitro. Moreover, the present stem-loop oligonucleotides bind target via both Watson-Crick and non-Watson-Crick hydrogen bonding. The present stem-loop oligonucleotides bind with strong affinity and high selectivity to their targeted nucleic acids.

Brief Summary Text (27):
In another embodiment the present invention provides a method of specific cell type drug delivery which includes administering an oligonucleotide of the present invention to an animal wherein the oligonucleotide has a covalently linked drug.

Detailed Description Text (2):
The present invention relates to stem-loop oligonucleotides which can bind to nucleic acid targets with high affinity and selectivity. Such strong, selective binding of these oligonucleotides to either single- or double-stranded DNA or RNA

targets provides a variety of uses, including methods of regulating such biological processes as DNA replication, RNA transcription, RNA splicing and processing and protein translation. Similarly, the strong binding properties of these stem-loop oligonucleotides makes these oligonucleotides ideal diagnostic probes or markers to localize, for example, specific sites in a chromosome or other DNA or RNA molecules. Additionally, the present stem-loop oligonucleotides are useful for isolation of complementary nucleic acids or for sequence-specific delivery of drugs or other molecules into cells.

Detailed Description Text (73):
The expression vectors of the present invention can also encode selectable markers. Selectable markers are genetic functions that confer an identifiable trait upon a host cell so that cells transformed with a vector carrying the selectable marker can be distinguished from non-transformed cells. Inclusion of a selectable marker into a vector can also be used to ensure that genetic functions linked to the marker are retained in the host cell population. Such selectable markers can confer any easily identified dominant trait, e.g. drug resistance, the ability to synthesize or metabolize cellular nutrients and the like.

Detailed Description Text (79):
Moreover, according to the present invention, the loop domains which do not encode a P or AP domain do not have to be composed of nucleotide bases. Non-nucleotide loop domains can make the present stem-loop oligonucleotides less expensive to produce. More significantly, stem-loop oligonucleotides with non-nucleotide loop domains are more resistent to nucleases and therefore have a longer biological half-life than linear oligonucleotides. Furthermore, non-nucleotide loop domains having no charge, or a positive charge, can be used to promote binding by eliminating negative charge repulsions between the loop and target. In addition, stem-loop oligonucleotides having uncharged or hydrophobic non-nucleotide loop domains can penetrate cellular membranes better than stem-loop oligonucleotides with nucleotide loops.

Detailed Description Text (86):
The present invention further contemplates derivatization of the subject oligonucleotides with agents that can cleave or modify the target nucleic acid or other nucleic acid strands associated with or in the vicinity of the target. For example, viral DNA or RNA can be targeted for destruction without harming cellular nucleic acids by administering a stem-loop oligonucleotide complementary to the targeted nucleic acid which is linked to an agent that, upon binding, can cut or render the viral DNA or RNA inactive. Nucleic acid destroying agents that are contemplated by the present invention as having cleavage or modifying activities include, for example, RNA and DNA nucleases, ribozymes that can cleave RNA, azidoproflavine, acridine, EDTA/Fe, chloroethylamine, azidophenacyl, psoralen and phenanthroline/Cu. Uhlmann et al. (1990, Chemical Reviews 90:543-584) and Beaucage et al. (1993, Tetrahedron 49:1925-1963) provide further information on the use of such agents and methods of derivatizing oligonucleotides that can be adapted for use with the subject stem-loop oligonucleotides.

Detailed Description Text (87):
Therefore, derivatization of the subject stem-loop oligonucleotides with reporter molecules, nucleic acid destroying agents, drugs, groups that facilitate cellular uptake or groups that facilitate target binding can be done by any of the procedures known to one skilled in the art. Moreover, the desired groups can be added to nucleotides before or after synthesis of the oligonucleotide. For example, these groups can be linked to the 5-position of T or C and these modified T and C nucleotides can be used for synthesis of the present stem-loop oligonucleotides.

Detailed Description Text (90):
These modifications can also increase the resistance of the subject oligonucleotides to nucleases. Methods for synthesis of oligonucleotides with modified phosphodiester linkages are reviewed by Uhlmann et al.

Detailed Description Text (93):
The present invention contemplates a variety of utilities for the subject stem-loop oligonucleotides which are made possible by their selective and stable binding properties with both single- and double-stranded targets. Some utilities include,

but are not limited to: regulating biosynthesis of a DNA, RNA or protein encoded by providing a stem-loop oligonucleotide to a template for the DNA, the RNA or the protein; use of stem-loop oligonucleotides of defined sequence, bound to a solid support, for affinity isolation of complementary nucleic acids; use of the subject oligonucleotides to provide sequence specific stop signals during polymerase chain reaction (PCR); covalent attachment of a drug, drug analog or other therapeutic agent to stem-loop oligonucleotides to allow cell type specific drug delivery; and labeling stem-loop oligonucleotides with a detectable reporter molecule for localizing, quantitating or identifying complementary target nucleic

Detailed Description Text (117):
Moreover, the present methods of regulating the biosynthesis of a DNA, RNA or protein can also be used to inhibit cellular oncogenes. Cellular protooncogenes are thought to have a normal role in cellular replication which is improperly executed when the protooncogene becomes mutated. Rather than controlling cellular growth, mutated protooncogenes, or oncogenes, can contribute to uncontrolled cellular growth and thereby increase the likelihood of developing cancer. The present methods can inhibit the transcription and translation of oncogenes such as, for example, oncogenes like the c-abl, bcr-abl, bcl-2, c-cbl, c-dbl, c-erb, c-ets, c-fgf, c-fms, c-fos, c-has/bas, her-2 neu, c-int, c-jun, c-kit, c-mas, c-met, c-mos, c-myb, c-myc, N-myc, p53, ras, c-Ha-ras, c-rel, c-ret, c-ros, c-sec, c-sis, c-ski, c-snoA, c-snoN, c-spi, c-src, c-syn, c-trk, c-vav and c-yes.

Detailed Description Text (121):
The present invention also contemplates using the subject stem-loop oligonucleotides for targeting drugs to specific cell types. Such targeting can allow selective destruction or growth of particular cell types, e.g. inhibition of tumor cell growth can be attained. To target a drug to a specific cell type the skilled artisan takes advantage of the fact that different cell types express different genes, so that the concentration of a particular mRNA can be greater in one cell type relative to another cell type. An mRNA which is present in higher concentrations in the cell to which the drug is to be delivered is a suitable target mRNA. Cells with high concentrations of target mRNA are targeted for drug delivery by administering to the cell a stem-loop oligonucleotide which is complementary to the target mRNA and which has a covalently linked drug.

Detailed Description Text (124):
Stem-loop oligonucleotide:solid supports can be used, for example, to isolate poly(A).sup.+ mRNA from total cellular or viral RNA when the stem-loop oligonucleotide has P and AP domain poly(dT) or poly(U) sequences. Stem-loop oligonucleotides are ideally suited to applications of this type because they are nuclease resistant and bind target nucleic acids so strongly.

Detailed Description Text (218):
Nuclease Resistance

Detailed Description Text (219):
The nuclease resistance of stem-loop and linear oligonucleotides are compared when these oligonucleotides are incubated in human plasma for varying time periods. Stem-loop and linear oligonucleotides having similar numbers of nucleotides are incubated at about a 50 .mu.M concentration in plasma at 37.degree. C. Aliquots are removed at various time points and cleavage products are separated by gel electrophoresis. Nuclease resistance is assessed by observing whether degradation products are evident on the gels.

Other Reference Publication (19):
Gewirtz et al. "Facilitating oligonucleotide delivery: Helping antisense deliver on its promise" Proc. Natl. Acad. Sci. USA 93: 3161-3163, Apr. 1996.

Other Reference Publication (20):
Miligan et al. "Current concepts in antisense drug design" J. Med. Chem. 36: 1923-1937, Jul. 1993.

Other Reference Publication (21):
Stull et al. "Antigene, ribozyme, and aptamer nucleic acid drugs; Progress and

prospects" Pharm. Res. 12: 465-483, Apr. 1995.

WEST

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L8: Entry 48 of 62 File: USPT Sep 29, 1998

DOCUMENT-IDENTIFIER: US 5814315 A
TITLE: Methods for the suppression of neu mediated phenotype in tumors

Brief Summary Text (6):
The c-erbB gene encodes the epidermal growth factor receptor (EGFr) and is highly homologous to the transforming gene of the avian erythroblastosis virus (Downward et al., 1984). The c-erbB gene is a member of the tyrosine-specific protein kinase family to which many proto-oncogenes belong. The c-erbB gene has recently been found to be similar, but distinct from, an oncogene referred to variously as c-erbB-2, HER-2 or neu oncogene (referred to herein simply as the neu oncogene), now known to be intimately involved in the pathogenesis of cancers of the human female breast and genital tract.

Brief Summary Text (64):
The term "E1A gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an E1A gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "E1A gene" may also comprise any combination of associated control sequences.

Brief Summary Text (68):
The term "LT gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an LT gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "LT gene" may also comprise any combination of associated control sequences.

Drawing Description Text (25):
(10A) E1A gene products inhibited the cell motility of the neu-transformed 3T3 cells. N-E1A: NIH3T3 cells transfected with E1A; B-neo: B104-1-1 cells transfected with neomycin resistant gene; B-E1A-1 to 5: five independent cell lines generated by transfecting E1A gene into B104-1-1 cells. The motility assays were carried out by using a transwell unit with 5 .mu.m pore size polycarbonate filter in 24 well cluster plate (Costar). Lower compartment of the transwell contained 600 .mu.l of one of the chemoattractants: 20 .mu.m fibronectin (FN) or 100 .mu.m FN dissolved in DMEM/F12, or hepatic endothelial cell conditioned media (HSE), or DMEM/F12 medial only as negative control. The cells (3.times.10.sup.4 /0.1 ml in DMEM/F12) were plated in the upper compartment and incubated for 6 hrs at 37.degree. C. in a humidified 5% CO.sub.2 atmosphere. After the incubation, the filters were fixed with 3% glutaraldehyde in PBS buffer and stained with Geimsa. ach sample was assayed in triplicate and cell motility was measured by counting the number of cells that had migrated to the lower side of the filter. At least four HPFs were counted per filter. The number of cells migrated to DMEM/F12 has been deducted from each sample to eliminate the background and all the assays were done in triplicates.

Drawing Description Text (55):
(20C) Effect of K1 on the transforming activity of activated neu. One mg of cNeu-104 was cotransfected with 2 mg of K1 and 0.1 mg of pSV2neo into Rat-1 cells. pSV2E was used as filler plasmid so that a final 5 mg DNA was transfected into cells. Cells were split 1:4 48 hours after transfection and duplicate plates were subsequently grown in regular medium (DMEM/F12 plus 10% calf serum) or regular medium supplemented with 250 mg/mL G418. Foci and G418-resistant colonies were stained and counted after 3-4 weeks. Results are expressed as ratio of foci to that of G418-resistant colonies from each transfection to correct for transfection

efficiency. The number of foci from transfecting cNeu-104 alone was set at 100%.

Detailed Description Text (3):
The activated neu oncogene contains a single amino acid substitution in the transmembrane domain and possesses an increased tyrosine kinase activity when compared to its normal counterpart. Furthermore, it has demonstrated that amplification of the neu protooncogene facilitates oncogenic activation by a single point mutation (Hung et al., 1989). The human homologue of the rat neu oncogene, also named as HER-2 or c-erbB2, has been shown to be amplified/overexpressed in 25-30% of human primary breast cancers and ovarian cancers (Hung et al., 1988; Slamon et al., 1987). Breast cancer patients with neu overexpression show a significantly lower overall survival rate and a shorter time to relapse than those patients without neu overexpression, suggesting that neu overexpression may be used as a prognostic factor (Id.). Amplification/overexpression of the human neu gene has also been shown to correlate with the number of axillary lymph nodes positive for metastasis in breast cancer patients (Id.). These studies strongly suggest that the neu oncogene may play an important role in malignant transformation and metastasis.

Detailed Description Text (50):
The B104-1-1 cell line, an NIH3T3 transfectant that has approximately 10-20 copies of mutation-activated genomic neu oncogene has been shown to be highly transforming and tumorigenic (Bargmann et al., 1986; Stern et al., 1986). For the present studies, B104-1-1 cells and control NIH3T3 cells were transfected with either E1A plasmids expressing adenovirus-5 E1A gene, (pE1A), or a derivative plasmid containing only the E1A promoter without the E1A coding sequence (pE1Apr). Cells were cotransfected with pSV2neo plasmids carrying a neomycin resistant marker gene (Southern et al., 1982).

Detailed Description Text (51):
The transfections were carried out with the modified calcium phosphate precipitation procedure of Chen and Okayama (1988). In each transfection, 5.times.10.sup.5 B104-1-1 cells or NIH3T3 cells (2.times.10 cm dishes) were seeded 24 h before transfection. The cells were transfected with either 10 .mu.g of the E1A expressing pE1A plasmas DNA or its derivative pE1Apr plasmid DNA, along with 1 .mu.g of pSV2-neo plasmid DNA (Southern et al., 1982). Approximately 14 h post-transfection, cells were washed and cultured in fresh medium for 24 h and split at a 1:10 ratio. The cells were then grown in selection medium containing 500 .mu.g/ml of G418 for 2-3 weeks and individual G418 resistant colonies were cloned using cloning rings and expanded to mass culture.

Detailed Description Text (74):
An increase in cell motility has been shown to correlate with a higher metastatic potential. Therefore, a motility assay, which measures the migration of the tested cell to a chemoattractant, fibronectin or hepatic sinusoidal endothelial cell conditioned media, was performed. As shown in FIG. 10A, all of the B-E1A transfectants showed decreased migration rate to different chemoattractants than that of B-neo cell line, which are B104-1-1 cells transfected with neomycin-resistant (neo.sup.r) gene alone. The N-E1A cells also had a low migration rate which is comparable to that of N1H3T3 cells.

Detailed Description Text (81):
The E1A-expressing plasmid was cotransfected into SKOV3.ip1 cells together with the pSV2-neo plasmid carrying the neomycin-resistance marker gene, thus generating the E1A-expressing ovarian carcinoma stable transfectants. The G418-resistant clones were selected and expanded into cell lines, which were designated ip1.E1A cell lines. The same approach was used to select control cell lines, in which the pE1Ad1343 plasmids containing a 2-base pair frameshift deletion in the E1A coding sequence and producing nonfunctional protein products were introduced into the SKOV3.ip1 cells to generate the ip1.Efs cell line.

Detailed Description Text (82):
It was possible that some of the stable transfectants selected by this cotransfection strategy only harbored the neomycin resistance gene but not the E1A gene. Therefore, to identify those ip1.E1A transfectants that integrated the E1A gene and actually produced E1A proteins, immunoblot analysis with anti-E1A

antibodies was performed (FIG. 12A). Two of the ip1.E1A transfectants expressed multiple species of E1A proteins as described by Harlow et al., (1985), whereas the control ip1.Efs cell line, as expected, did not express E1A proteins.

Detailed Description Text (107):
NIH 3T3, B104-1-1 and Rat-1 cells were maintained in 5% C02 in Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 10% calf serum and 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells transfected with the drug selection plasmid, pSV2neo, were grown in the above media containing 400 mg/mL G418.

Detailed Description Text (111):
The drug selection plasmid pSV2neo was cotransfected with plasmids encoding LT into B104-1-1 cells. The transfected plates were trypsinized after 48 h and split into 4 plates and subsequently maintained in media containing 400 mg/mL G418. After 3 weeks, colonies were isolated and established in media containing G418).

Detailed Description Text (119):
Focus forming assay was carried out as described (Yu et al., 1992). The cosmid clone, cNeu-104 (Hung et al., 1986), contains 30 kb of activating genomic rat neu including 2.2 kb of the neu promoter. cNeu-104 (0.5 mg) was cotransfected into normal fibroblasts (Rat-1 cells) with 0.1 mg of the drug selection plasmid, pSV2neo, and 5-10 mg plasmids encoding mutant LT (pK1) or control filler plasmid, pSV2E. Cells were trypsinized and split into 4 plates 48 h after transfection. Two plates were maintained in regular media while the other 2 plates were maintained in media supplemented with G418. For cells kept in regular media for 3 weeks, foci of transformed cells appeared on a background monolayer of nontransformed cells. G418 resistant colonies appeared for plates maintained in G418 media. Foci and G418 resistant colonies were stained with 1% crystal violet and counted. To normalize for transfection efficiency, the number of foci formed for each transfection was divided by the number of G418 colonies obtained.

Detailed Description Text (122):
To test the effect of LT in cells that overexpress neu encoded p185, plasmids encoding LT, pZ189 (driven by the SV 40 promoter), together with pSV2neo (plasmids encoding the gene for neomycin resistance) were cotransfected into B104-1-1 cells. B104-1-1 cells are derived from NIH 3T 3 cells transformed by the mutation-activated genomic rat neu oncogene (Shih et al., 1981; Hung et al., 1986). B104-1-1 cells express high levels of activated neu encoded p185, are phenotypically transformed (Padhy et al., 1982; Shih et al., 1981), highly tumorigenic (Yu et al., 1991; Hung et al. 1989) and have increased metastatic potential (Yu et al., 1991; Yu et al. 1992). The LT-transfected and G418 resistant B104-1-1 cells were cloned after 3 weeks and 2 cell lines expanded from the clones (named BTn14 and BTn16 cell lines were analyzed for expression of LT and p185. Immunoblotting of cell lysates for LT using anti-LT antibody (SV 40 T-Ag, Ab-2, Oncogene Science), showed 2 bands of molecular weights less than 111 kd indicating expression of LT in BTn14 and BTn16 cell lines (FIG. 15-B, lanes 1 and 2). The bands are probably different phosphorylated forms of LT, as reported previously (Livingston et al., 1987). A control cell line, BEn5, was generated by transfecting B104-1-1 cells with pSV2neo and pSV2E (control plasmid similar to pZ189, containing the SV 40 promoter but lacking the LT coding region). As expected, BEn5 and NIH 3T3 cells do not express LT (FIG. 15-B, lanes 3 and 4).

Detailed Description Text (160):
These results indicate that liposome-mediated E1A gene transfer can inhibit neu-overexpressing human ovarian cancer cell growth. Therefore, it is predictable that liposome-mediated E1A or LT gene therapy may serve as a powerful therapeutic agent for HER-2 neu-overexpressing human ovarian cancers by direct targeting of E1A or LT at the HER-2 neu-oncogene.

Other Reference Publication (38):
Felgner et al., "Gene Therapeutics: The Direct Delivery of Purified Genes in vivo and Their Application as Drugs, Without the use of Retroviruses, Is Discussed," Nature, 349:351-352 (1991).

L8: Entry 45 of 62 File: USPT Nov 24, 1998

DOCUMENT-IDENTIFIER: US 5840525 A
TITLE: Nucleic acids, vectors and host cells encoding heregulin

Detailed Description Text (49):
HRG also includes NTD-GFD having its C-terminus at one of the first about 1 to 3 extracellular domain residues (QKR, residues 240-243, HRE-.alpha., FIG. 15) or first about 1-2 transmembrane region residues. In addition, in some HRG-GFD variants the codons are modified at the GFD-transmember proproteolysis site by substitution, insertion or deletion. The GFD proteolysis site is the domain that contains the GFD C-terminal residue and about 5 residues N- and 5 residues C-terminal from this residue. At this time neither the natural C-terminal residue for HRG-.alpha. or HRG-.beta. has been identified, although it is known that Met-227 terminal and Val-229 terminal HRG-.alpha.-GFD are biologically active. The native C-terminus for HRG-.alpha.-GFD is probably Met-227, Lys-228, Val-229, Gln-230, Asn-231 or Gln-232, and for HRG .beta..sub.1 -.beta..sub.2- GFD is probably Met-226, Ala-227, Ser-228, Phe-229, Trp-230, Lys 231 or (for HRG-.beta..sub.1) K240 or (for HRG-.beta..sub.2) K246. The native C-terminus Is determined readily by C-terminal sequencing, although it is not critical that HRG-GFD have the native terminus so long as the GFD sequence possesses the desired activity. In some embodiments of HRG-GFD variants, the amino acid change(s) in the CTP are screened for their ability to resist proteolysis in vitro and inhibit the protease responsible for generation of HRG-GFD.

Detailed Description Text (116):
Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Detailed Description Text (117):
One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1: 327,1982), mycophenolic acid (Mulligan et al., Science 209: 1422,1980) or hygromycin (Sugden et al., Mol. Cell. Biol. 5: 410-413, 1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Detailed Description Text (119):
For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216, 1980. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR

gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding HRG. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding HRG, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418 (see U.S. Pat. No. 4,965,199).

Detailed Description Text (134):
For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res. 9: 309 (1981) or by the method of Maxam et al., Methods in Enzymology 65: 499 (1980).

Detailed Description Text (147):
The mammalian host cells used to produce HRG of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (IMEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58: 44 (1979), Barnes and Sato, Anal. Biochem., 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or copending U.S. Ser. Nos. 07/592,107 or 07/592,141, both filed on 3 Oct. 1990, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin.TM. drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Detailed Description Text (168):
HRG optionally is fused with a polypeptide heterologous to HRG. The heterologous polypeptide optionally is an anchor sequence such as that found in the decay accelerating system (DAF); a toxin such as ricin, pseudomonas exotoxin, gelonin, or other polypeptide that will result in target cell death. These heterologous polypeptides are covalently coupled to HRG through side chains or through the terminal residues. Similarly, HRG is conjugated to other molecules toxic or inhibitory to a target mammalian cell, e.g. such as tricothecenes, or antisense DNA that blocks expression of target genes.

Detailed Description Text (177):
HRG also is entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

Detailed Description Text (204):
Antibodies that are capable of binding to proteolytic processing sites are of particular interest. They are produced either by immunizing with an HRG fragment that includes the CTP processing site, with intact HRG, or with HRG-NTD-GFD and then

screening for the ability to block or inhibit proteolytic processing of HRG into the NTD-GFD fragment by recombinant host cells or isolated cell lines that are otherwise capable of processing HRG to the fragment. These antibodies are useful for suppressing the release of NTD-GFD and therefore are promising for use in preventing the release of NTD-GFD and stimulation of the HER-2 receptor. They also are useful in controlling cell growth and replication. Anti-GFD antibodies are useful for the same reasons, but may not be as efficient biologically as antibodies directed against a processing site.

Detailed Description Text (205):
Antibodies are selected that are capable of binding only to one of the members of the HRG family, e.g. HRG-alpha or any one of the HRG-beta isoforms. Since each of the HRG family members has a distinct GFD-transmembrane domain cleavage site, antibodies directed specifically against these unique sequences will enable the highly specific inhibition of each of the GFDs or processing sites, and thereby refine the desired biological response. For example, breast carcinoma cells which are HER-2 dependent may in fact be activated only by a single GFD isotype or, if not, the activating GFD may originate only from a particular processing sequence, either on the HER-2 bearing cell itself or on a GFD-generating cell. The identification of the target activating GFD or processing site is a straight-forward matter of analyzing HER-2 dependent carcinomas, e.g., by analyzing the tissues for the presence of a particular GFD family member associated with the receptor, or by analyzing the tissues for expression of an HRG family member (which then would serve as the therapeutic target). These selective antibodies are produced in the same fashion as described above, either by immunization with the target sequence or domain, or by selecting from a bank of antibodies having broader specificity.

Detailed Description Text (206):
As described above, the antibodies should have high specificity and affinity for the target sequence. For example, the antibodies directed against GFD sequences should have greater affinity for the GFD than GFD has for the HER-2 receptor. Such antibodies are selected by routine screening methods.

Detailed Description Text (283):
Heregulin-.beta.2 and -.beta.3 variants were isolated in order to obtain cDNA clones that extend further in the 5' direction. A specifically primed cDNA library was constructed in .lambda.gt10 by employing the chemically synthesized antisense primer 3' CCTTCCCGTTCTTCTTCCTCGCTCC (SEQ ID NO: 21). This primer is located between nucleotides 167-190 in the sequence of .lambda.her16 (FIG. 4). The isolation of clone .lambda.5'her13 (not to be confused with .lambda.her13) was achieved by hybridizing a synthetic DNA probe corresponding to the 5' end of .lambda.her16 under high stringency conditions with the specifically primed cDNA library. The nucleotide sequence of .lambda.5'her13 is shown in FIG. 11 (SEQ ID NO: 22). The 496 base pair nucleotide sequence of .lambda.5'her13 is homologous to the sequence of .lambda.her16 between nucleotides 309-496 of .lambda.5'her13 and 3-190 of .lambda.her16. .lambda.5'her13 extends by 102 amino acids the open reading frame of .lambda.her16.

L8: Entry 37 of 62 File: USPT Dec 14, 1999

DOCUMENT-IDENTIFIER: US 6001583 A
TITLE: Methods for disrupting GRB-7 complexes

Detailed Description Text (4):
Second, methods are described for the identification of such agents. These methods may include, for example, assays to identify agents capable of disrupting or inhibiting the interaction between components of the protein tyrosine kinase/adaptor protein complexes, and may also include paradigms and strategies for the rational design of drugs capable of disruption and/or inhibition of such complexes.

Detailed Description Text (44):
Techniques for decreasing the cellular level and/or the activity of one or more of the PTK/adaptor protein complex components of interest may include, but are not limited to antisense or ribozyme approaches, and/or gene therapy approaches, each of which is well known to those of skill in the art, as described, below, in Sections 5.2.2.1-5.2.2.2.

Detailed Description Text (45):
5.2.2.1 ANTISENSE AND RIBOZYME APPROACHES

Detailed Description Text (46):
Included in the scope of the invention are oligoribonucleotides, including antisense RNA and DNA molecules and ribozymes that function to inhibit translation of one or more components of a PTK/adaptor protein complex. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the PTK nucleotide sequence of interest, are preferred. In the case of GRB-7, for example, this sequence would correspond to nucleotides 359 through 379 of the GRB-7 DNA sequence.

Detailed Description Text (47):
Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding PTK/adaptor protein complex components.

Detailed Description Text (48):
Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Detailed Description Text (49):
Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared

by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Detailed Description Text (55):
The particular compound, antibody, antisense or ribozyme molecule that affects the PTK/adaptor protein complexes and the oncogenic disorders of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

Detailed Description Text (96):
The PTK/adaptor complex of the present invention may be biochemically purified from a variety of cell or tissue sources. For purification of a naturally occurring PTK/adaptor complex, cellular sources may include, for example, baculovirus-infected SF9 cells, A-431, CHO, and/or 3T3 cells. In a preferred embodiment of the present invention, the PTK/adaptor complex comprises the receptor PTK HER2 and the GRB-7 adaptor protein. Sources for the purification of such a HER2/GRB-7 complex may include, but are not limited to the SKBR-3 cell line (ATCC HTB30). Other sources could include, for example, BT474 (ATCC HTB20) cells, a cell line transfected with nucleotide sequences to express both HER-2 and GRB-7, ZR-75-30 (ATCC CRL1504) cells, MDA-MB-453 (ATCC HTB27) cells, or tumors extracted from transgenic mice expressing both GRB-7 and HER-2.

Detailed Description Text (106):
For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably coexpress both the PT and adaptor protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the PTK and adaptor protein DNA independently or coordinately controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which coexpress both the PTK and adaptor protein. Such engineered cell lines are particularly useful in screening and evaluation of compounds that affect signals mediated by the complexes.

Detailed Description Text (107):
A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk, hgprt or aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

CLAIMS:

4. A method for disrupting a HER-2 receptor protein tyrosine kinase polypeptide/GRB-7 adaptor polypeptide complex comprising, contacting a cell that forms the complex with a compound that acts intracellularly at a concentration

sufficient to disrupt the HER-2 receptor protein tyrosine kinase polypeptide/GRB-7 complex.

L8: Entry 35 of 62 File: USPT Dec 21, 1999

DOCUMENT-IDENTIFIER: US 6004940 A
TITLE: Intracellular targeting of endogenous proteins

Brief Summary Text (7):
Therapeutic strategies to combat conditions caused by undesired expression of a protein have included the development of drugs to target the undesired proteins, means of intercellular blocking of such proteins, for example, the use of an antibody to the protein on the cell surface, and the use of drugs which will selectively kill cells expressing the undesired proteins.

Brief Summary Text (9):
Modem molecular techniques which inhibit the expression of specific genes provide methods for the manipulation of individual genes and cellular pathways. For example, techniques using antisense RNA, ribozymes, dominant negative mutants and targeted gene disruption have all been successfully used to inhibit the expression or function of specific genes. [Neckers, L. & Whitesell, L., Am. J. Physiol. 245:11-12 (1993); Erickson, R. P., Dev. Gene. 14:251-257 (1993); Bratty, J., et al., Biochimica et Biophysica Acta. 1261:345-359 (1993); Schaap, D., et al., J. Biol. Chem. 268:20232-20236 (1993); Capecchi, M. R., Sci. Am. 270-52-58 (1994)]. However, these methods have certain limitations. The dominant negative mutant approach, for example, requires the availability of a functionally inactive mutant which acts in a dominant manner to suppress the activity of the wild type protein. Procedures using antisense RNA produce inhibitory effects that are frequently incomplete or short lived [Neckers, L. & Whitesell, L., Am. J. Physiol. 245:11-12 (1993); Erickson, R. P., Dev. Gene. 14:251-257 (1993); Nellen, W., et al., TIBS 18:419-423 (1993)]. A major impediment to the development of effective gene inhibition protocols using antisense RNA or ribozymes is the inability to achieve a high level of expression of the inhibitor encoding DNA template in the transformed cells. This may also be a potential problem with using dominant negative mutants because of the competitive nature of the inhibition. Additionally, the targeted gene disruption technique is limited because this method cannot readily be applied to cells en masse.

Brief Summary Text (18):
The capacity to disrupt the expression of specific IL-2 receptor chains in cells such as primary lymphocytes and established cell lines can have both therapeutic value and utility for drug screening. For example, an anti-IL-2 antibody (referred to as a Tac antibody) can be used. Preferably, one uses a single chain antibody (sFv). More preferably, one uses a Tac antibody with a specific cellular localization sequence. For example, one preferred cellular location to target is the endoplasmic reticulum (ER). A preferred localization sequence is the KDEL sequence. Preferred single-chain intrabodies of this invention, hereinafter referred to as "sFvTac", "sFvTacKDEL", can be used to down regulate IL-2R.alpha.. No toxicity is associated with intracellular expression of the IL-2R.alpha. intrabodies in cells that do not require IL-2 R.alpha. for viability, and the transport of other plasma membrane proteins is unaffected. The intrabodies of this invention are efficient and specific and are useful in regulating receptors, such as IL-2 receptors. The stable introduction of genes encoding single-chain intrabodies, therefore, provides a powerful alternative to antisense RNA, ribozymes and other methods of gene inactivation.

Detailed Description Text (51):
Both intrabodies are expressed inside cells. However, the sFv Tac KDEL intrabody is

retained in the ER, whereas, the sFv Tac intrabody continues to move through the cell. As a consequence, the two intrabodies bind to and form complexes at different intracellular sites. For example, the ER intrabody (sFvTacKDEL) binds and holds the receptor chain in the ER. For example, the sFvTacKDEL intrabody coprecipitates with IL-2R.alpha., suggesting a physical interaction between the two proteins inside the cell. Furthermore, IL-2R.alpha. is detectable within these cells as an immature 40 kD form that is sensitive to endoglycosidase-H. See FIG. 6, lanes 4 and 4. The absence of complex carbohydrates, which are added in the golgi and would render the protein resistant to endo H, thereby show that the 40 kD precursor is held in an early golgi or pre-golgi compartment. FIG. 6.

Detailed Description Text (76):
For expression in mammalian cells, the assembled sFvTac gene was reamplified using primers A and H or A and I. The H primer introduces a stop codon at the end of V.sub.L. The I primer introduces six additional amino acids (SEKDEL) at the C-terminus, followed by a stop codon. The amplified fragments were digested with HindIII and XbaI and ligated into the vector pRc/CMV (Invitrogen). The resulting plasmids, designated pCMVTac and pCMVTacKDEL, contain the sFvTac gene under the control of the cytomegalovirus immediate early promoter and a neo gene, conferring resistance to G418.

Detailed Description Text (78):
pCMVTac and pCMVTacKDEL were linearized at an XmnI site within the .beta.-lactamase gene and introduced into Jurkat cells by electroporation. 1.times.10.sup.7 cells were pulsed with 10 .mu.g of DNA (300 V, 960 .mu.F) using a Gene Pulser (Biorad). G418 selection was applied at 0.8 mg/ml after 36 hours. Two weeks later, G418 resistant cells were seeded at 0.3 cell per well in 96-well plates using 25% conditioned medium.

Detailed Description Text (94):
In common with most secreted and cell surface proteins, newly synthesized IL-2R.alpha. undergoes extensive post-translational processing in the ER and golgi. The protein has 2 potential N-linked glycosylation sites and carbohydrate accounts for almost half of the molecular mass of the mature receptor chain. SDS-PAGE analysis of IL-2R.alpha. immunoprecipitated from C8166 or PHA/PMA-treated Jurkat cells revealed 2 forms: a predominant 55 kD form (p55), representing the mature receptor, and a less abundant 40 kD form (p40) which has previously been identified as a an immature form of IL-2R.alpha.. [Wano, Y., et al. J. Immunol. 132:3005-3010 (1984)]. FIG. 6, lane 1. Pulse chase experiments confirmed the 40 kD protein to be a natural precursor of p55, which is chased into the higher molecular weight form within two hours in control Jurkat cells. FIG. 7. PHA/PMA treatment of Jurkat cells expressing sFvTacKDEL was associated with intracellular accumulation of the p40 precursor and the complete absence of p55 (FIG. 6, lane 3 and 4b). Moreover, a 30 kD protein identical in size to the single-chain antibody coprecipitated with p40 IL-2R.alpha. in these cells (FIGS. 6 and 7). The p40 form of IL-2R.alpha. was sensitive to endoglycosidase H digestion and is therefore a high mannose glycoprotein (FIG. 6 lanes 1-4). The absence of complex oligosaccharides, which are added in the medial golgi and render the glycoprotein resistant to endo-H, suggests that p40 is located in a pre-golgi or early golgi compartment. As expected, both the mature p55 (FIG. 6, lanes 1 and 2) and soluble Tac a form of IL-2R.alpha. that is shed into the extracellular fluid, (not shown) were insensitive to endo H digestion. These results indicate that IL-2R.alpha. is translocated into the ER and core glycosylated in cells expressing sFvTacKDEL, but is unable to proceed to the golgi owing to its stable association with the ER-localized intrabody.

Detailed Description Paragraph Table (1):
TABLE 1.sup.1 _____ HOMOLOGOUS CATEGORY ONCOGENE
CELLULAR GENE _____ Growth Factors sis PDGF-/2
int-2 FGF-like Transmembrane erbB EGF receptor growth factors neu (erbB-2, HER-2)
fms M-CSF receptor ros, kit, and others Membrane-associated abl tyrosinc kinases
Membrane associated src family.sup.2 guanine nucleotide fes, fps.sup.3 binding
proteins K-, N- and H-ras Cytoplasmic serine- raf/mil threonine kinases mos
Cytoplasmid hormone erbA Thyroid hormone receptor receptors Nuclear factors c-myc,
N-myc, L-myc, fos, jun, myb, ets, ski, and others Antioncogenes RB Others bcl-2
bcl-1 int-1 _____ .sup.1 Adapted from Druker, B.

J., et al., N. Eng. J. of Mol. 321:1383-1392 (1989). PDGF denotes plateletderived growth factor, FGF fibroblast growth factor, EGF epidermal growth factor, and MCSF mononuclearphagocyte growth factor. .sup.2 The family includes src, fgr, yes, lck, hck, fyn, lyn, and tkl. .sup.3 The subcellular location of these oncongene products is uncertain.

L8: Entry 33 of 62 File: USPT Mar 14, 2000

DOCUMENT-IDENTIFIER: US 6037134 A
TITLE: Methods that detect compounds that disrupt receptor tyrosine kinase/GRB-7 complexes

Brief Summary Text (17):
5.2.2.1 ANTISENSE AND RIBOZYME APPROACHES . . .

Detailed Description Text (4):
Second, methods are described for the identification of such agents. These methods may include, for example, assays to identify agents capable of disrupting or inhibiting the interaction between components of the protein tyrosine kinase/adaptor protein complexes, and may also include paradigms and strategies for the rational design of drugs capable of disruption and/or inhibition of such complexes.

Detailed Description Text (44):
Techniques for decreasing the cellular level and/or the activity of one or more of the PTK/adaptor protein complex components of interest may include, but are not limited to antisense or ribozyme approaches, and/or gene therapy approaches, each of which is well known to those of skill in the art, as described, below, in Sections 5.2.2.1-5.2.2.2.

Detailed Description Text (45):
5.2.2.1 ANTISENSE AND RIBOZYME APPROACHES

Detailed Description Text (46):
Included in the scope of the invention are oligoribonucleotides, including antisense RNA and DNA molecules and ribozymes that function to inhibit translation of one or more components of a PTK/adaptor protein complex. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the PTK nucleotide sequence of interest, are preferred. In the case of GRB-7, for example, this sequence would correspond to nucleotides 359 through 379 of the GRB-7 DNA sequence.

Detailed Description Text (47):
Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding PTK/adaptor protein complex components.

Detailed Description Text (48):
Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using

ribonuclease protection assays.

Detailed Description Text (49):
Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Detailed Description Text (55):
The particular compound, antibody, antisense or ribozyme molecule that affects the PTK/adaptor protein complexes and the oncogenic disorders of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

Detailed Description Text (95):
The PTK/adaptor complex of the present invention may be biochemically purified from a variety of cell or tissue sources. For purification of a naturally occurring PTK/adaptor complex, cellular sources may include, for example, baculovirus-infected SF9 cells, A-431, CHO, and/or 3T3 cells. In a preferred embodiment of the present invention, the PTK/adaptor complex comprises the receptor PTK HER2 and the GRB-7 adaptor protein. Sources for the purification of such a HER2/GRB-7 complex may include, but are not limited to the SKBR-3 cell line (ATCC HTB30). Other sources could include, for example, BT474 (ATCC HTB20) cells, a cell line transfected with nucleotide sequences to express both HER-2 and GRB-7, ZR-75-30 (ATCC CRL1504) cells, MDA-MB-453 (ATCC HTB27) cells, or tumors extracted from transgenic mice expressing both GRB-7 and HER-2.

Detailed Description Text (105):
For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably coexpress both the PT and adaptor protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the PTK and adaptor protein DNA independently or coordinately controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which coexpress both the PTK and adaptor protein. Such engineered cell lines are particularly useful in screening and evaluation of compounds that affect signals mediated by the complexes.

Detailed Description Text (106):
A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk.sup.-, hgprt.sup.- or aprt.sup.- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

L8: Entry 10 of 62 File: USPT May 28, 2002

DOCUMENT-IDENTIFIER: US 6395712 B1
TITLE: Sensitization of HER-2/neu overexpressing cancer cells to chemotherapy

Abstract Text (1):
The present invention relates to methods for the inhibition, of the gene product of the neu oncogene, p185neu tyrosine kinase. Over-expression of the neu oncogene leads to chemoresistance. The methods disclosed involve the novel use of E1A and/or LT in combination with chemotherapeutic drugs to treat carcinoma. Furthermore, E1A surprisingly potentiates the antineoplastic effects of the chemotherapeutic agents. The inventors propose that E1A sensitizes cancer cells such that they become amenable to treatment by chemotherapeutic drugs.

Brief Summary Text (22):
Thus the present invention, in a general and overall sense, concerns methods of inhibiting oncogene-mediated transformation of a cell and sensitizing the cell to chemotherapeutic agents using gene products. These methods involve contacting the cell with a neu-suppressing gene product and a chemotherapeutic drug in amounts effective to inhibit the transformed phenotype.

Brief Summary Text (26):
Some embodiments of the invention involve methods of inhibiting oncogene-mediated transformation of a cell. Generally, these methods comprise the step of contacting the cell with an oncogenic phenotype suppressing gene product and a chemotherapeutic drug in amounts effective to inhibit the transformed phenotype. In a preferred embodiment, the oncogene-mediated transformation being inhibited will be neu oncogene-mediated transformation. Also, preferably, the embodiments in which transformation is to be inhibited will comprises a tyrosine specific protein kinase encoded by neu. Of course, the invention also applies to methods of inhibiting other oncogene-mediated transformation events, such as transformation by ras, src, yes, fps, fes, abl, ros, fgr, erbB, fms, mos, raf etc.

Brief Summary Text (27):
Embodiments of the present invention involve chemotherapeutic agents. These are compounds that exhibit some form of anti-cancer activity. In some preferred embodiments, the chemotherapeutic drug is an alkylating agent, plant alkaloid, antibiotic, or antineoplastic agent. In those embodiments of the invention where the chemotherapeutic is an alkylating agent, the alkylating agent may be, for example, mechlorethamine, cyclophosphamide, ifosfamide chlorambucil, melphalan, busulfan, thiotepa, carmustine, lomustine, and/or streptozocin. In those embodiments where the chemotherapeutic agent comprises a plant alkaloid, the plant alkaloid is, for example, vincristine, vinblastine or taxol. In a preferred embodiment, the plant alkaloid is taxol. In those embodiments of the invention where the chemotherapeutic agent is an antibiotic, the antibiotic may be, for example, dactinomycin, daunorubicin, idarubicin, bleomycin mitomycin or doxorubicin. In most preferred embodiments the antibiotic is doxorubicin. In other embodiments where the chemotherapeutic agent comprises an antineoplastic, the preferred antineoplastic is, for example, cisplatin, VP16 and TNF.

Brief Summary Text (28):
In certain embodiments of the invention, the E1A or LT is administered to the cell prior to the administration of the chemotherapeutic agent. In other aspects of the invention, the chemotherapeutic agent is administered to the cell prior to

administration of the E1A or LT. Alternatively the E1A or LT and the chemotherapeutic drug are administered simultaneously.

Brief Summary Text (29):
In some embodiments of the invention, the cell is located within an animal and effective amounts of the E1A or LT and the chemotherapeutic drug are administered to the animal. In certain embodiments of the invention, the chemotherapeutic drug and the E1A or LT are suitably dispersed in a pharmacologically acceptable formulation. In certain preferred embodiments where the cell is an animal cell, the animal cell is a human cell. In other preferred embodiments the cells is a lung, cancer cell, ovarian cancer cell, or a breast cancer cell.

Brief Summary Text (31):
The invention contemplates embodiments comprising sensitizing a cancer cell to a chemotherapeutic drug. These embodiments comprise exposing the cell with an effective amount of the E1A or LT. In some such embodiments inhibition of neu-mediated cancer is accomplished by administrating an effective combination of the E1A or LT and chemotherapeutic drug to an animal having or suspected of having cancer in an effective amount to inhibit the cancer. In embodiments where the composition is administered to an animal, the animal is typically a mammal. In such cases, the invention will be of particular use in the treatment and prevention of neu-mediated transformation in humans

Brief Summary Text (32):
Certain embodiments of the present invention comprise injecting a therapeutically effective amount of the E1A or LT into an animal and contacting the animal with a chemotherapeutic drug. In certain embodiments of the invention the cancer site is contacted with a chemotherapeutic drug by administering to the animal a therapeutically effective amount of a pharmaceutical composition comprising a chemotherapeutic drug wherein the chemotherapeutic drug is for example cisplatin, doxorubicin, VP16, taxol or TNF.

Brief Summary Text (33):
The inventors have also enabled the production of pharmaceutical compositions comprising an E1A or LT and a chemotherapeutic drug in a pharmacological carrier. Those of skill will understand the nature of such pharmacological carriers based on the teachings of this specification and the current knowledge in the art. The pharmaceutical compositions of the invention may contain any of the E1A or LT and chemotherapeutic drugs mentioned above or elsewhere in this specification, or know to those of skill in the art. They may also contain emodin and/or an emodin like compound. In a preferred pharmaceutical composition the chemotherapeutic drug is cisplatin, doxorubicin, etoposide, taxol or TNF. In some preferred embodiments, the neu-suppressing gene product is E1A. In some preferred embodiments the neu-suppressing gene product is LT.

Brief Summary Text (34):
The invention also encompasses pharmaceutical combinations comprising an a neu-suppressing gene product and a chemotherapeutic drug. In certain preferred combinations, the neu-suppressing gene product is E1A. In certain other preferred combinations, the neu-suppressing gene product is LT. The chemotherapeutic drug may be any that is listed elsewhere in this specification or known to those of skill in the art at the present or in the future. Exemplary chemotherapeutic drugs for us in the pharmaceutical combinations of the present invention are cisplatin, doxorubicin, etoposide, emodin and or emodin like compounds, taxol and TNF. In certain embodiments of the invention the pharmaceutical combination may contain the E1A or LT and the chemotherapeutic drug within the same pharmaceutical composition. In other embodiments, the pharmaceutical combinations will comprise separate pharmaceutical compositions for each of the E1A or LT and the chemotherapeutic drug. These separate compositions may be combined internal to or external to a body to create the pharmaceutical combination.

Brief Summary Text (35):
Other embodiments of the invention include therapeutic kits comprising in suitable container, a pharmaceutical formulation of an the E1A or LT preparation, a pharmaceutical formulation of a chemotherapeutic drug, and/or a pharmaceutical

formulation comprising both the E1A or LT and a chemotherapeutic drug. Emodin and/or emodin like compounds may also be present in the kit either in combination with the gene products, chemotherapeutic agent, gene products and chemotherapeutic agent or indeed in a separate formulation. The kit may also contain instructions on how to administer the pharmaceutical formulation or formulations of the kit to an animal either alone, or in combination with formulations that one may obtain separately from the kit. The kit may also comprise instructions that explain how to use the kit but are provided separately from the container of the kit. The kit may comprise the E1A or LT, emodin and/or emodin like compound, and chemotherapeutic drug to be present within a single container or alternatively the kit could comprise the E1A or LT and/or emodin and the chemotherapeutic drug are present within distinct containers.

Brief Summary Text (36):
Some embodiments of the present invention relate to a method of sensitization of a cell to an anticancer drug, comprising contacting the cell with the E1A or LT. These gene products are well-described in this specification. In preferred embodiments the oncogene-mediated transformation is neu oncogene-mediated transformation. Also, preferably, the embodiments in which transformation is to be inhibited will comprises a tyrosine specific protein kinase encoded by neu. The invention also contemplates pharmaceutical compositions, and kits comprising the E1A or LT to suppress neu-mediated transformation. Of course, the invention also applies to methods of inhibiting or suppressing other oncogene-mediated transformation events, such as transformation by ras, src, yes, fps, fes, abl, ros, fgr, erbB, fms, mos, raf.

Brief Summary Text (38):
Other embodiments of the present invention relate to a method of inhibiting oncogene-mediated transformation of a cell, comprising contacting the cell with the E1A or LT, further contacting the cell with emodin and/or an emodin-like compound and further still contacting the cell with the chemotherapeutic agent. The cell may be contacted with the gene product, the emodin and/or emodin like compound and the chemotherapeutic agent successively in any order. Alternatively the cell is contacted with a combination of gene product and emodin, gene product and chemotherapeutic drug, emodin and chemotherapeutic drug followed or preceded by treatment of the third agent. In yet another embodiment it is possible to contact the cell with the gene product, emodin and/or emodin like compound and chemotherapeutic agent concurrently with each other.

Drawing Description Text (27):
FIG. 10A E1A gene products inhibited the cell motility of the neu-transformed 3T3 cells. N-E1A: NIH3T3 cells transfected with E1A; B-neo: B104-1-1 cells transfected with neomycin resistant gene; B-E1A-1 to 5: five independent cell lines generated by transfecting E1A gene into B 104-1-1 cells. The motility assays were carried out by using a transwell unit with 5 .mu.m pore size polycarbonate filter in 24 well cluster plate (Costar). Lower compartment of the transwell contained 600 .mu.l of one of the chemoattractants: 20 .mu.m fibronectin (FN) or 100 .mu.m FN dissolved in DMEM/F12, or hepatic-endothelial cell conditioned media (HSE), or DMEM/F12 medial only as negative control. The cells (3.times.10.sup.4 /0.1 ml in DMEM/F12) were plated in the upper compartment and incubated for 6 hrs. at 37.degree. C. in a humidified 5% CO.sub.2 atmosphere. After the incubation, the filters were fixed with 3% glutaraldehyde in PBS buffer and stained with Geimsa. Each sample was assayed in triplicate and cell motility was measured by counting the number of cells that had migrated to the lower side of the filter. At least four HPFs were counted per filter. The number of cells migrated to DMEM/F12 has been deducted from each sample to eliminate the background and all the assays were done in triplicates.

Drawing Description Text (57):
FIG. 20C. Effect of K1 on the transforming activity of activated neu. One mg of cNeu-104 was cotransfected with 2 mg of K1 and 0.1 mg of pSV2neo into Rat-1 cells. pSV2E was used as filler plasmid so that a final 5 mg DNA was transfected into cells. Cells were split 1:4 48 hours after transfection and duplicate plates were subsequently grown in regular medium (DMEM/F12 plus 10% calf serum) or regular medium supplemented with 250 mg/mL G418. Foci and G418-resistant colonies were stained and counted after 3-4 weeks. Results are expressed as ratio of foci to that

of G418-resistant colonies from each transfection to correct for transfection efficiency. The number of foci from transfecting cNeu-104 alone was set at 100%.

Detailed Description Text (3):
The invention provides methods for treating neu-mediated cancers using a neu-suppressing gene product and a chemotherapeutic drug in order to inhibit neu-tyrosine kinase activity. The methods of the invention generally rest in using genes, for example, the E1A or the LT gene in combination with an anti-cancer agent effective to treat the cancer cells associated with neu over-expression.

Detailed Description Text (7):
The term "E1A gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an E1A gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "E1A gene" may also comprise any combination of associated control sequences.

Detailed Description Text (11):
The term "LT gene" refers to any DNA sequence that is substantially identical to a DNA sequence encoding an LT gene product as defined above. The term also refers to RNA, or antisense sequences compatible with such DNA sequences. An "LT gene" may also comprise any combination of associated control sequences.

Detailed Description Text (46):
Administered alone, it is the drug of first choice for the treatment of thyroid adenoma and primary hepatocellular carcinoma. It is a component of 31 first-choice combinations for the treatment of ovarian, endometrial and breast tumors, bronchogenic oat-cell carcinoma, non-small cell lung carcinoma, gastric adenocarcinoma, retinoblastoma, neuroblastoma, mycosis fungoides, pancreatic carcinoma, prostatic carcinoma, bladder carcinoma, myeloma, diffuse histiocytic lymphoma, Wilms' tumor, Hodgkin's disease, adrenal tumors, osteogenic sarcoma soft tissue sarcoma, Ewing's sarcoma, rhabdomyosarcoma and acute lymphocytic leukemia. It is an alternative drug for the treatment of islet cell, cervical, testicular and adrenocortical cancers. It is also an immunosuppressant.

Detailed Description Text (48):
Appropriate doses are, intravenous, adult, 60 to 75 mg/m.sup.2 at 21-day intervals or 25 to 30 mg/m.sup.2 on each of 2 or 3 successive days repeated at 3- or 4-wk intervals or 20 mg/m.sup.2 once a week. The lowest dose should be used in elderly patients, when there is prior bone-marrow depression caused by prior chemotherapy or neoplastic marrow invasion, or when the drug is combined with other myelopoietic suppressant drugs. The dose should be reduced by 50% if the serum bilirubin lies between 1.2 and 3 mg/dL and by 75% if above 3 mg/dL. The lifetime total dose should not exceed 550 mg/m.sup.2 in patients with normal heart function and 400 mg/m.sup.2 in persons having received mediastinal irradiation. Alternatively, 30 mg/m.sup.2 on each of 3 consecutive days, repeated every 4 wk. Exemplary doses may be 10 mg/m.sup.2, 20 mg/m.sup.2, 30 mg/m.sup.2, 50 mg/m.sup.2, 100 mg/m.sup.2, 150 mg/m.sup.2, 175 mg/m.sup.2, 200 mg/m.sup.2, 225 mg/m.sup.2, 275 mg/m.sup.2, 300 mg/m.sup.2, 350 mg/m.sup.2, 400 mg/m.sup.2, 425 mg/m.sup.2, 450 mg/m.sup.2, 500 mg/m.sup.2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Detailed Description Text (52):
In combination with other drugs it is included in the first-choice chemotherapy of acute myelocytic leukemia in adults (for induction of remission), acute lymphocytic leukemia and the acute phase of chronic myelocytic leukemia. Oral absorption is poor, and it must be given intravenously. The half-life of distribution is 45 minutes and of elimination, about 19 hr. The half-life of its active metabolite, daunorubicinol, is about 27 hr. Daunorubicin is metabolized mostly in the liver and also secreted into the bile (ca 40%). Dosage must be reduced in liver or renal insufficiencies.

Detailed Description Text (56):
Mitomycin selectively inhibits the synthesis of deoxyribonucleic acid (DNA). The guanine and cytosine content correlates with the degree of mitomycin-induced cross-linking. At high concentrations of the drug, cellular RNA and protein

synthesis are also suppressed.

Detailed Description Text (60):
Actinomycin D (Dactinomycin) [50-76-0]; C.sub.62 H.sub.86 N.sub.12 O.sub.16 (1255.43) is an antineoplastic drug that inhibits DNA-dependent RNA polymerase. It is a component of first-choice combinations for treatment of choriocarcinoma, embryonal rhabdomyosarcoma, testicular tumor and Wilms' tumor. Tumors which fail to respond to systemic treatment sometimes respond to local perfusion. Dactinomycin potentiates radiotherapy. It is a secondary (efferent) immunosuppressive.

Detailed Description Text (61):
Actinomycin D is used in combination with primary surgery, radiotherapy, and other drugs, particularly vincristine and cyclophosphamide. Antineoplastic activity has also been noted in Ewing's tumor, Kaposi's sarcoma, and soft-tissue sarcomas. Dactinomycin can be effective in women with advanced cases of choriocarcinoma. It also produces consistent responses in combination with chlorambucil and methotrexate in patients with metastatic testicular carcinomas. A response may sometimes be observed in patients with Hodgkin's disease and non-Hodgkin's lymphomas. Dactinomycin has also been used to inhibit immunological responses, particularly the rejection of renal transplants.

Detailed Description Text (62):
Half of the dose is excreted intact into the bile and 10% into the urine; the half-life is about 36 hr. The drug does not pass the blood-brain barrier. Actinomycin D is supplied as a lyophilized powder (0/5 mg in each vial). The usual daily dose is 10 to 15 mg/kg; this is given intravenously for 5 days; if no manifestations of toxicity are encountered, additional courses may be given at intervals of 3 to 4 weeks. Daily injections of 100 to 400 mg have been given to children for 10 to 14 days; in other regimens, 3 to 6 mg/kg, for a total of 125 mg/kg, and weekly maintenance doses of 7.5 mg/kg have been used. Although it is safer to administer the drug into the tubing of an intravenous infusion, direct intravenous injections have been given, with the precaution of discarding the needle used to withdraw the drug from the vial in order to avoid subcutaneous reaction. Exemplary doses may be 100 mg/m.sup.2, 150 mg/m.sup.2, 175 mg/m.sup.2, 200 mg/m.sup.2, 225 mg/m.sup.2, 250 mg/m.sup.2, 275 mg/m.sup.2, 300 mg/m.sup.2, 350 mg/m.sup.2, 425 mg/m.sup.2, 450 mg/m.sup.2, 475 mg/m.sup.2, 500 mg/m.sup.2. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Detailed Description Text (79):
VP16 is available as a solution (20 mg/ml) for intravenous administration and as 50-mg, liquid-filled capsules for oral use. For small-cell carcinoma of the lung, the intravenous dose (in combination therapy) is can be as much as 100 mg/m.sup.2 or as little as 2 mg/m.sup.2, routinely 35 mg/m.sup.2, daily for 4 days, to 50 mg/m.sup.2, daily for 5 days have also been used. When given orally, the dose should be doubled. Hence the doses for small cell lung carcinoma may be as high as 200-250 mg/m.sup.2. The intravenous dose for testicular cancer (in combination therapy) is 50 to 100 mg/m.sup.2 daily for 5 days, or 100 mg/m.sup.2 on alternate days, for three doses. Cycles of therapy are usually repeated every 3 to 4 weeks. The drug should be administered slowly during a 30- to 60-minute infusion in order to avoid hypotension and bronchospasm, which are probably due to the solvents used in the formulation.

Detailed Description Text (81):
Tumor Necrosis Factor [TNF; Cachectin] is a glycoprotein that kills some kinds of cancer cells, activates cytokine production, activates macrophages and endothelial cells, promotes the production of collagen and collagenases, is an inflammatory mediator and also a mediator of septic shock, and promotes catabolism, fever and sleep. Some infectious agents cause tumor regression through the stimulation of TNF production. TNF can be quite toxic when used alone in effective doses, so that the optimal regimens probably will use it in lower doses in combination with other drugs. Its immunosuppressive actions are potentiated by gamma-interferon, so that the combination potentially is dangerous. A hybrid of TNF and interferon-.alpha. also has been found to possess anti-cancer activity.

Detailed Description Text (86):
Vincristine blocks mitosis and produces metaphase arrest. It seems likely that most of the biological activities of this drug can be explained by its ability to bind specifically to tubulin and to block the ability of protein to polymerize into microtubules. Through disruption of the microtubules of the mitotic apparatus, cell division is arrested in metaphase. The inability to segregate chromosomes correctly during mitosis presumably leads to cell death.

Detailed Description Text (87):
The relatively low toxicity of vincristine for normal marrow cells and epithelial cells make this agent unusual among anti-neoplastic drugs, and it is often included in combination with other myelosuppressive agents.

Detailed Description Text (88):
Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM.

Detailed Description Text (90):
Vincristine has a multiphasic pattern of clearance from the plasma; the terminal half-life is about 24 hours. The drug is metabolized in the liver, but no biologically active derivatives have been identified. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Detailed Description Text (91):
Vincristine sulfate is available as a solution (1 mg/ml) for intravenous injection. Vincristine used together with corticosteroids is presently the treatment of choice to induce remissions in childhood leukemia; the optimal dosages for these drugs appear to be vincristine, intravenously, 2 mg/m.sup.2 of body-surface area, weekly, and prednisone, orally, 40 mg/m.sup.2, daily. Adult patients with Hodgkin's disease or non-Hodgkin's lymphomas usually receive vincristine as a part of a complex protocol. When used in the MOPP regimen, the recommended dose of vincristine is 1.4 mg/m.sup.2. High doses of vincristine seem to be tolerated better by children with leukemia than by adults, who may experience sever neurological toxicity. Administration of the drug more frequently than every 7 days or at higher doses seems to increase the toxic manifestations without proportional improvement in the response rate. Precautions should also be used to avoid extravasation during intravenous administration of vincristine. Vincristine (and vinblastine) can be infused into the arterial blood supply of tumors in doses several times larger than those that can be administered intravenously with comparable toxicity.

Detailed Description Text (95):
When cells are incubated with vinblastine, dissolution of the microtubules occurs. Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM. Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes.

Detailed Description Text (96):
After intravenous injection, vinblastine has a multiphasic pattern of clearance from the plasma; after distribution, drug disappears from plasma with half-lives of approximately 1 and 20 hours.

Detailed Description Text (98):
Vinblastine sulfate is available in preparations for injection. The drug is given intravenously; special precautions must be taken against subcutaneous extravasation, since this may cause painful irritation and ulceration. The drug should not be injected into an extremity with impaired circulation. After a single dose of 0.3 mg/kg of body weight, myelosuppression reaches its maximum in 7 to 10 days. If a moderate level of leukopenia (approximately 3000 cells/mm.sup.3) is not attained, the weekly dose may be increased gradually by increments of 0.05 mg/kg of body weight. In regimens designed to cure testicular cancer, vinblastine is used in doses of 0.3 mg/kg every 3 weeks irrespective of blood cell counts or toxicity.

Detailed Description Text (104):
Although it is generally agreed that carmustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

Detailed Description Text (105):
Carmustine is indicated as palliative therapy as a single agent or in established combination therapy with other approved chemotherapeutic agents in brain tumors such as glioblastoma, brainstem glioma, medullobladyoma, astrocytoma, ependymoma, and metastatic brain tumors. Also it has been used in combination with prednisone to treat multiple myeloma. Carmustine has proved useful, in the treatment of Hodgkin's Disease and in non-Hodgkin's lymphomas, as secondary therapy in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

Detailed Description Text (106):
The recommended dose of carmustine as a single agent in previously untreated patients is 150 to 200 mg/m.sup.2 intravenously every 6 weeks. This may be given as a single dose or divided into daily injections such as 75 to 100 mg/M.sup.2 on 2 successive days. When carmustine is used in combination with other myelosuppressive drugs or in patients in whom bone marrow reserve is depleted, the doses should be adjusted accordingly. Doses subsequent to the initial dose should be adjusted according to the hematologic response of the patient to the preceding dose. It is of course understood that other doses may be used in the present invention for example 10 mg/m.sup.2, 20 mg/m.sup.2, 30 mg/m.sup.2 40 mg/m.sup.2 50 mg/m.sup.2 60 mg/m.sup.2 70 mg/m.sup.2 80 mg/m.sup.2 90mg/m.sup.2 100 mg/m.sup.2. The skilled artisan is directed to, "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

Detailed Description Text (110):
Available evidence suggests that about one third to one half of the patients with multiple myeloma show a favorable response to oral administration of the drug.

Detailed Description Text (115):
Suitable doses for adults include, orally, 1 to 5 mg/kg/day (usually in combination), depending upon gastrointestinal tolerance; or 1 to 2 mg/kg/day; intravenously, initially 40 to 50 mg/kg in divided doses over a period of 2 to 5 days or 10 to 15 mg/kg every 7 to 10 days or 3 to 5 mg/kg twice a week or 1.5 to 3 mg/kg/day A dose 250 mg/kg/day may be administered as an antineoplastic. Because of gastrointestinal adverse effects, the intravenous route is preferred for loading. During maintenance, a leukocyte count of 3000 to 4000/mm.sup.3 usually is desired. The drug also sometimes is administered intramuscularly, by infiltration or into body cavities. It is available in dosage forms for injection of 100, 200 and 500 mg, and tablets of 25 and 50 mg the skilled artisan is referred to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61, incorporate herein as a reference, for details on doses for administration.

Detailed Description Text (118):
Chlorambucil is available in tablet form for oral administration. It is rapidly and completely absorbed from the gastrointestinal tract. After single oral doses of 0.6-1.2 mg/kg, peak plasma chlorambucil levels are reached within one hour and the terminal half-life of the parent drug is estimated at 1.5 hours. 0.1 to 0.2 mg/kg/day or 3 to 6 mg/m.sup.2 /day or alternatively 0.4 mg/kg may be used for antineoplastic treatment. Treatment regimes are well know to those of skill in the art and can be found in the "Physicians Desk Reference" and in "Remingtons Pharmaceutical Sciences" referenced herein.

Detailed Description Text (126):
Although it is generally agreed that lomustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in

proteins.

Detailed Description Text (129):
Lomustine has been shown to be useful as a single agent in addition to other treatment modalities, or in established combination therapy with other approved chemotherapeutic agents in both primary and metastatic brain tumors, in patients who have already received appropriate surgical and/or radiotherapeutic procedures. It has also proved effective in secondary therapy against Hodgkin's Disease in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

Detailed Description Text (130):
The recommended dose of lomustine in adults and children as a single agent in previously untreated patients is 130 mg/m.sup.2 as a single oral dose every 6 weeks. In individuals with compromised bone marrow function, the dose should be reduced to 100 mg/m.sup.2 every 6 weeks. When lomustine is used in combination with other myelosuppressive drugs, the doses should be adjusted accordingly. It is understood that other doses may be used for example, 20 mg/m.sup.2 30 mg/m.sup.2, 40 mg/m.sup.2, 50 mg/m.sup.2, 60 mg/m.sup.2, 70 mg/m.sup.2, 80 mg/m.sup.2, 90 mg/m.sup.2, 100 mg/m.sup.2, 120 mg/m.sup.2 or any doses between these FIG.s as determined by the clinician to be necessary for the individual being treated.

Detailed Description Text (141):
The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. The kits of the invention may also include an instruction sheet defining administration of the gene therapy and/or the chemotherapeutic drug.

Detailed Description Text (151):
Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

Detailed Description Text (162):
One of the preferred methods for in vivo delivery involves the use of an adenovirus vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized. Adenoviral transfer of E1A is especially useful, because E1A is itself an adenoviral gene. Therefore, there need be no non-viral genetic sequences inserted into an adenoviral vector to accomplish adenoviral delivery of E1A. Of course, LT-encoding DNA, or other neu-suppressing gene product encoding sequences may be introduced via adenoviral vectors as well.

Detailed Description Text (197):
In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished in vitro, as in laboratory procedures for transforming cells lines, or in vivo or ex vivo (see below), as in the treatment of certain disease states. As described above, delivery may be via viral infection where the expression construct is encapsidated in an infectious viral particle.

Detailed Description Text (250):
The B104-1-1 cell line, an NIH3T3 transfectant that has approximately 10-20 copies of mutation-activated genomic neu oncogene has been shown to be highly transforming and tumorigenic (Bargmann et al., 1986; Stern et al., 1986). For the present studies, B104-1-1 cells and control NIH3T3 cells were transfected with either E1A plasmids expressing adenovirus-5 E1A gene, (pE1A), or a derivative plasmid containing only the E1A promoter without the E1A coding sequence (pE1Apr). Cells were cotransfected with pSV2neo plasmids carrying a neomycin resistant marker gene

(Southern et al., 1982).

Detailed Description Text (251):
The transfections were carried out with the modified calcium phosphate precipitation procedure of Chen and Okayama (1988). In each transfection, 5.times.10.sup.5 B104-1-1 cells or NIH3T3 cells (2.times.10 cm dishes) were seeded 24 h before transfection. The cells were transfected with either 10 .mu.g of the E1A expressing pE1A plasmid DNA or its derivative pE1Apr plasmid DNA, along with 1 .mu.g of pSV2-neo plasmid DNA (Southern et al., 1982). Approximately 14 h post-transfection, cells were washed and cultured in fresh medium for 24 h and split at a 1:10 ratio. The cells were then grown in selection medium containing 500 .mu.g/ml of G418 for 2-3 weeks and individual G418 resistant colonies were cloned using cloning rings and expanded to mass culture.

Detailed Description Text (274):
An increase in cell motility has been shown to correlate with a higher metastatic potential. Therefore, a motility assay, which measures the migration of the tested cell to a chemo-attractant, fibronectin or hepatic sinusoidal endothelial cell conditioned media, was performed. As shown in FIG. 10A, all of the B-E1A transfectants showed decreased migration rate to different chemoattractants than that of B-neo cell line, which are B104-1-1 cells transfected with neomycin-resistant (neo') gene alone. The N-E1A cells also had a low migration rate which is comparable to that of N1H3T3 cells.

Detailed Description Text (281):
The E1A-expressing plasmid was cotransfected into SKOV3.ip1 cells together with the pSV2-neo plasmid carrying the neomycin-resistance marker gene, thus generating the E1A-expressing ovarian carcinoma stable transfectants. The G418-resistant clones were selected and expanded into cell lines, which were designated ip1.E1A cell lines. The same approach was used to select control cell lines, in which the pE1Ad1343 plasmids containing a 2-base pair frameshift deletion in the E1A coding sequence and producing nonfunctional protein products were introduced into the SKOV3.ip1 cells to generate the ip1.Efs cell line.

Detailed Description Text (282):
It was possible that some of the stable transfectants selected by this cotransfection strategy only harbored the neomycin resistance gene but not the E1A gene. Therefore, to identify those ip1.E1A transfectants that integrated the E1A gene and actually produced E1A proteins, immunoblot analysis with anti-E1A antibodies was performed (FIG. 12A). Two of the ip1.E1A transfectants expressed multiple species of E1A proteins as described by Harlow et al., (1985), whereas the control ip1.Efs cell line, as expected, did not express E1A proteins.

Detailed Description Text (307):
NIH 3T3, B104-1-1 and Rat-1 cells were maintained in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 10% calf serum and 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells transfected with the drug selection plasmid, pSV2neo, were grown in the above media containing 400 mg/mL G418.

Detailed Description Text (311):
The drug selection plasmid pSV2neo was cotransfected with plasmids encoding LT into B104-1-1 cells. The transfected plates were trypsinized after 48 h and split into 4 plates and subsequently maintained in media containing 400 mg/mL G418. After 3 weeks, colonies were isolated and established in media containing G418).

Detailed Description Text (319):
Focus forming assay was carried out as described (Yu et al., 1992). The cosmid clone, cNeu-104 (Hung et al., 1986), contains 30 kb of activating genomic rat neu including 2.2 kb of the neu promoter. cNeu-104 (0.5 mg) was cotransfected into normal fibroblasts (Rat-1 cells) with 0.1 mg of the drug selection plasmid, pSV2neo, and 5-10 mg plasmids encoding mutant LT (pK1) or control filler plasmid, pSV2E. Cells were trypsinized and split into 4 plates 48 h after transfection. Two plates were maintained in regular media while the other 2 plates were maintained in media supplemented with G418. For cells kept in regular media for 3 weeks, foci of transformed cells appeared on a background monolayer of nontransformed cells. G418

resistant colonies appeared for plates maintained in G418 media. Foci and G418 resistant colonies were stained with 1% crystal violet and counted. To normalize for transfection efficiency, the number of foci formed for each transfection was divided by the number of G418 colonies obtained.

Detailed Description Text (322):
To test the effect of LT in cells that overexpress neu encoded p185, plasmids encoding LT, pZ189 (driven by the SV 40 promoter), together with pSV2neo (plasmids encoding the gene for neomycin resistance) were cotransfected into B104-1-1 cells. B104-1-1 cells are derived from NIH 3T3 cells transformed by the mutation-activated genomic rat neu oncogene (Shih et al., 1981; Hung et al., 1986). B104-1-1 cells express high levels of activated nest encoded p185, are phenotypically transformed (Padhy et al., 1982; Shih et al., 1981), highly tumorigenic (Yu et al., 1991; Hung et al. 1989) and have increased metastatic potential (Yu et al., 1991; Yu et al. 1992). The LT-transfected and G418 resistant B104-1-1 cells were cloned after 3 weeks and 2 cell lines expanded from the clones (named BTn14 and BTn16 cell lines) were analyzed for expression of LT and p185. Immunoblotting of cell lysates for LT using anti-LT antibody (SV 40 T-Ag, Ab-2, Oncogene Science), showed 2 bands of molecular weights less than 111 kd indicating expression of LT in BTn14 and BTn16 cell lines (FIG. 15-B, lanes 1 and 2). The bands are probably different phosphorylated forms of LT, as reported previously (Livingston et al., 1987). A control cell line, BEn5, was generated by transfecting B104-1-1 cells with pSV2neo and pSV2E (control plasmid similar to pZ189, containing the SV 40 promoter but lacking the LT coding region). As expected, BEn5 and NIH 3T3 cells do not express LT (FIG. 15-B, lanes 3 and 4).

Detailed Description Text (361):
These results indicate that liposome-mediated E1A gene transfer can inhibit neu-overexpressing human ovarian cancer cell growth. Therefore, it is predictable that liposome-mediated E1A or LT gene therapy may serve as a powerful therapeutic agent for HER-2 neu-overexpressing human ovarian cancers by direct targeting of E1A or LT at the HER-2 neu-oncogene.

Detailed Description Text (394):
These studies are based on the discovery that Ad.E1A has tumor suppressor activity for neu-overexpressing cancer cells. The Examples above further show that Ad.E1A inhibits the growth of neu-mediated cancer cells and furthermore sensitizes neu-mediated cancer cells to chemotherapeutic drugs. The current example uses of either Ad.E1A or LT, in combination with chemotherapeutic drugs, to provide a useful preventive and therapeutic regimen for patients with neu-overexpressing cancers.

Detailed Description Text (395):
Two groups of mice of a suitable cancer model will be treated with doses of E1A or LT in combination with anti cancer drugs starting at 6 weeks of age. Several combinations and concentrations of E1A or LT and anti-cancer drugs will be tested. Control mice will be treated with buffer only.

Detailed Description Text (396):
The effect of E1A or LT, in combination with an anticancer drug, on the development of breast tumors will be compared with the control group by examination of tumor size, p185.sup.neu tyrosine kinase activity (using IP-western blot analysis) and histopathologic examination (breast tissue will be cut and stained with hematoxylin and eosin) of breast tissue. With the chemopreventive potential of E1A and LT, it is predicted that, unlike the control group of mice that develop tumors, the testing group of mice will be resistant to tumor development.

Detailed Description Text (399):
These mice may then be given an appropriate dosage of E1A or LT using methods of delivery described above; in combination with an anti-cancer drug for 3 consecutive days, then once a week for six months.

Detailed Description Text (402):
Five days after treatment with the p185-overexpressing cells, mice may be separated into control and experimental groups. One group of mice will be left untreated. Other groups will be treated. Active compounds may be supplied to a treated group in

phosphate buffer saline. One treated group will be treated with the buffered saline only. Another treated group may receive treatment with an appropriate dosage of E1A or LT. A third treated group may be treated with an appropriate dosage of an anti-cancer drug alone. A final group may be treated with an appropriate dosage of E1A or LT in combination with an anti-cancer drug. Treatments may be given using any of the methods described above.

Detailed Description Text (403):
Mice may be examined for tumor signs and symptoms, and killed when they appear moribund. Mice treated with the E1A or LT plus the anti-cancer drug will be expected to have a longer survival time.

Detailed Description Text (405):
In order to obtain mice with the human lung cell cancer, nu/nu mice may be given as intratracheal injections of, for example, 2.times.10.sup.6 viable neu overexpressing cancer cells from cell line H82. Five days after inoculation, following tumor formation, mice may be separated into groups to begin treatment. One group may be treated with an appropriate dosage of E1A or LT alone, another with an appropriate dosage of an anti-cancer drug alone. A third group may be treated with an appropriate dosage of E1A or LT in combination with an anticancer drug for 3 consecutive days, then once a week for two months.

Detailed Description Text (407):
Human Treatment With E1A or LT in Combination With Anti-cancer Drugs or Alone

Detailed Description Text (408):
This example describes a protocol to facilitate the treatment of neu-mediated cancer using E1A or LT in combination with anti-cancer drugs.

Detailed Description Text (417):
A major challenge in clinical oncology of neu-mediated cancers is that tumor cells over-expressing the neu-protooncogene are resistant to chemotherapeutic treatment. One goal of the inventors' efforts has been to find ways to improve the efficacy of chemotherapy. In the context of the present invention, E1A or LT can be combined with any of a number of conventional chemotherapeutic regimens.

Detailed Description Text (426):
Clinical Trials of the Use of E1A or LT in Combination With Anti-Cancer Drugs in Treating Neu-Mediated Cancer

Detailed Description Text (427):
This example is concerned with the development of human treatment protocols using the E1A and LT in combination with anti-cancer drugs. E1A or LT and anti-cancer drug treatment will be of use in the clinical treatment of various neu-overexpressing cancers in which transformed or cancerous cells play a role. Such treatment will be particularly useful tools in anti-tumor therapy, for example, in treating patients with ovarian, breast and lung cancers that are mediated by neu over-expression and resistant to conventional chemotherapeutic regimens.

Detailed Description Text (428):
The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing E1A or LT in combinations with anti-cancer drugs in clinical trials.

Detailed Description Text (430):
In regard to the E1A or LT and other anti-cancer drug administration, a Tenokhoff catheter, or alternative device may be placed in the pleural cavity or in the peritoneal cavity, unless such a device is already in place from prior surgery. A sample of pleural or peritoneal fluid can be obtained, so that baseline cellularity, cytology, LDH, and appropriate markers in the fluid (CEA, CA15-3, CA 125, p185) and in the cells (E1A, p185) may be assessed and recorded.

Detailed Description Text (431):

In the same procedure, E1A or LT may be administered alone or in combination with the anti-cancer drug. The administration may be in the pleural/peritoneal cavity, directly into the tumor, or in a systemic manner. The starting dose may be 0.5 mg/kg body weight. Three patients may be treated at each dose level in the absence of grade >3 toxicity. Dose escalation may be done by 100% increments (0.5 mg, 1 mg, 2 mg, 4 mg) until drug related grade 2 toxicity is detected. Thereafter dose escalation may proceed by 25% increments. The administered dose may be fractionated equally into two infusions, separated by six hours if the combined endotoxin levels determined for the lot of E1A or LT and the lot of anti-cancer drug exceed 5EU/kg for any given patient.

Detailed Description Text (432):
The E1A or LT and anti-cancer drug combination may be administered over a short infusion time or at a steady rate of infusion over a 7 to 21 day period. The E1A or LT infusion may be administered alone or in combination with the anti-cancer drug. The infusion given at any dose level will be dependent upon the toxicity achieved after each. Hence, if Grade II toxicity was reached after any single infusion, or at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of E1A or LT in combination with an anti-cancer drug will be administered to groups of patients until approximately 60% of patients show unacceptable Grade III or IV toxicity in any category. Doses that are 2/3 of this value could be defined as the safe dose.

Other Reference Publication (28):
Yu et al., "Overexpression of c-erB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1 independent mechanisms," Oncogene, 13:1359-1365, 1996.

Other Reference Publication (42):
Zhang and Hung, "Sensitization of HER-2/neu-overexpressing Non-Small Cell Lung Cancer Cells to Chemotherapeutic Drugs by tyrosine kinase Inhibitor Emodin," Oncogene 12:571-576, 1996.

Other Reference Publication (49):
Friche et al., "Effect of anthracycline analogs on photolabelling of p-glycoprotein by [125I]iodomycin and [3H]azidopine: relation to lipophilicity and inhibition of daunorubicin transport in multidrug resistant cells," Br. J. Cancer, 67(2):226-231, 1993.

Other Reference Publication (59):
Hudziak et al., "Amplified Expression of the the HER2/ERBB2 Oncogene Induces Resistance to Tumor Necrosis Factor a in NIH 3T3 Cells," Proc. Natl. Acad. Sci. USA, 85:5102-5106, 1988.

Other Reference Publication (79):
Lichtenstein et al., "Resistance of Human Ovarian Cancer Cells to Tumor Necrosis Factor and Lymphokine-Activated Killer Cells: Correlation with Expression of HER2/neu Oncogenes," Cancer Research, 50:7364-7370, 1990.

Other Reference Publication (108):
Shepard, H. M. and G. D. Lewis, "Resistance of Tumor Cells to Tumor Necrosis Factor," J. of Clin. Immunol. 8(5):333-341, 1988.

Other Reference Publication (130):
Chevalier, Fumoleau, Kerbrat, Dieras, Roche, Krakowski, Azli, Bayssas, Lentz, Van Glabbeke, "Decetaxel is a major cytotoxic drug for the treatment of advanced breast cancer: a phase II trial of the Clinical Screening Cooperative Group of the European Organization for Research and Treatment of Cancer," J Clin. Oncol., 13:314-322, 1995.

Other Reference Publication (135):
Felgner et al., "Gene Therapeutics: The Direct Delivery of Purified Genes in vivo and Their Application as Drugs, Without the Use of Retroviruses, Is Discussed," Nature, 349:351-352 (1991).